RIINA KUUSELO

MED29 Possesses a Complex Role in Pancreatic Cancer

ACADEMIC DISSERTATION
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YHTEENVETO

Haimasyöpä on yksi huonoennusteisimpia syöpiä, johon sairastuu ja kuolee Suomessa vuosittain lähes 900 ihmistä. Nykyiset syöpälääkkeet tehoavat haimasyöpään heikosti ja taudin ennuste on erittäin huono. Duktaalinen adenokarsinooma on haimasyövän yleisin muoto ja termi ’haimasyöpä’ viittaa tässä työssä nimenomaan duktaaliseen alatyyppiin. Tässä tutkimuksessa pyrittiin kartoittamaan haimasyövän molekyylitason muutoksia ja löytämään uusia geenejä, joita voitaisiin tulevaisuudessa hyödyntää taudin diagnostiikassa ja hoidossa. Tutkimus keskittyi erityisesti kromosomissa 19 sijaitsevaan 19q13-monistuma-alueeseen, tässä lokuksessa geenimonistuman kautta aktivoituvien geenien tunnistamiseen ja niiden toiminnalliseen karakterisointiin.

Työn ensimmäisessä vaiheessa hyödynnettiin fluoresenssi *in situ* hybridisaatio -menetelmää, jonka avulla kartoitettiin 19q13 alueen kopiolukumuutoksia haimasyöpäsolulinjoissa. Tämän tutkimuksen avulla pystyttiin rajaamaan 1,1 Mb:n suuruinen monistuma-alue. Rajatun alueen sisältä löytyi vielä 600 kb:n kokoinen ydinalue, jossa monistuman taso oli huomattavan suuri. Seuraavaksi monistuman yleisyys määritettiin primaareissa haimakasvaimissa, ja sen sen mahdollista yhteyttä kliinis-patologisiin tekijöihin tutkittiin laajassa yli 500 potilaan aineistossa. Monistuman havaittiin esiintyvän yli 10%:ssa haiman adenokarsinoomia, ja mikä tärkeintä, sen huomattiin korreloivan kasvaimen huonon erilaistumisasteen ja taudin levinnäisyyden kanssa. Monistuma ei suoranaisesti ollut yhteydessä potilaiden eloonjäämiseen, mutta potilaiden, joilla esiintyi korkea-asteista 19q13 monistumaa, keskimääräinen elinaika oli selvästi lyhyempi kuin niiden, joilla kyseistä monistumaa ei ollut.

Monistuma-alueella sijaitsevat geenit tunnistettiin julkisten tietokantojen ja ihmisen genomikartoituksen tarjoaman tiedon avulla, ja niiden ilmentymistasot määritettiin kvantitatiivista reaaliaikaista PCR-tekniikkaa hyödyntäen. Tämä analyysi osoitti, että useat alueella sijaitsevat geenit ilmentyivät voimakkaammin monistuman myötä. Seuraavaksi tutkittiin näiden geenien hiljentämisen vaikutusta

haimasyöpäsolujen elinvoimaisuuteen RNA-inhibitio (RNAi) -menetelmän avulla. Geenit, joiden hiljentäminen heikensi solujen elinkykyä, luokiteltiin mahdollisiksi monistuman kohdegeeneiksi. *MED29*-geeni (*IXL*) osoittautui näissä kokeissa vahvimmaksi kandidaatiksi ja oli myös voimakkaasti yli-ilmentynyt monistuman omaavissa soluissa, joten jatkotutkimukset keskittyivät siihen.

RNA-inhibitioon perustuvaa geenin hiljentämistä hyödynnettiin haimasyöpäsoluissa, joissa *MED29*:n ilmentymistaso oli sisäsyntyisesti korkea. Hiljentäminen johti useiden syöpäsoluille tyypillisten ominaisuuksien, kuten solukasvun ja solujen migraatio- ja invaasiokyvyn, heikentymiseen. Lentivirus-pohjainen *MED29*-geenin yli-ilmentäminen haimasyöpäsoluissa, joissa se ei luonnollisesti ilmentynyt, johti yllättäen myös kasvun hidastumiseen. Merkittävin löydös oli kuitenkin se, että näiden solujen istutus immuunipuutteiseen hiireen johti dramaattiseen kasvainten määrän ja koon pienenemiseen kontrollisoluihin verrattuna, viitaten selvästi kasvua rajoittavaan ominaisuuteen.

Mikrosirupohjaista genominlaajuista tutkimusmenetelmää käytettiin hyväksi selvittämään mekanismeja, jotka aiheuttivat kasvuerot MED29- ja kontrollisolujen välillä. Tutkimus toi esiin suuren joukon erilaisesti ilmentyviä geenejä, jotka geeniontologiatutkimuksen perusteella osallistuvat mm. solusyklin ja solunjakautumisen säätelyyn. Yleisesti ottaen mikrosirututkimus paljasti, että MED29 vaikuttaa useiden solusykliä säätelevien geenien toimintaan tavalla, joka johtaa solujakautumisen pysähtymiseen.

Yhteenvetona voidaan todeta, että 19q13-kromosomialueen monistuma on kliinisesti merkittävä ja se korreloi kasvaimen erilaistumis- ja levinnäisyysasteeseen haimasyövässä. Korkea-asteinen 19q13 monistuma johtaa useiden geenien ilmentymisen lisääntymiseen, mutta *MED29* vaikuttaa tällä hetkellä olevan yksi biologisesti merkittävimmistä monistuman kohdegeeneistä. *MED29*-geenin hiljentäminen rajoitti solun kasvua, elinkykyä ja muita syöpäsolulle tunnusomaisia piirteitä haimasyöpäsoluissa, joissa se oli monistunut. Soluissa, joissa *MED29*-geenin monistumaa ei ollut, johti sen ilmentäminen myös heikentyneeseen solukasvuun ja rajoitti huomattavasti tuumorikasvua hiireen istutetuissa siirrännäisissä. MED29 on osa transkription säätelyyn osallistuvaa mediaattorikompleksia, jonka on hiljattain osoitettu olevan osallisena useassa syövässä. Tämä tutkimus osoittaa nyt ensimmäistä kertaa, että MED29:llä on merkittävä, mutta kompleksinen rooli haimasyövän patogeneesissä.

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following communications, which are referred to in the text by their Roman numerals.

I Kuuselo R, Savinainen K, Azorsa DO, Basu GD, Karhu R, Tuzmen S, Mousses S, Kallioniemi A. Intersex like (IXL) is a cell survival regulator in pancreatic cancer with 19q13 amplification. Cancer Res, 67:1943-1949, 2007

II Kuuselo R, Simon R, Karhu R, Tennstedt P, Marx AH, Izbicki JR, Yekebas E, Sauter G, Kallioniemi A. 19q13 amplification associates with high grade and stage in pancreatic cancer. Genes Chrom Cancer, 49:569-575, 2010.

III Kuuselo R[1], Savinainen K[1], Sandström S, Autio R, Kallioniemi A. MED29, a component of the Mediator complex, possesses both oncogenic and tumor suppressive characteristics in pancreatic cancer. Submitted.

[1]Authors contributed equally to this work

ABBREVIATIONS

aCGH	array comparative genomic hybridization
ABL	v-abl Abelson murine leukemia viral oncogene homolog 1
ADP	adenosine diphosphate
AIB1	amplified in breast cancer 1
AKT2	v-akt murine thymoma viral oncogene homolog 2
ALL	acute lymphoblastic leukemia
ANAPC4	anaphase promoting complex subunit 4
AP-1	activator protein 1
APC	adenomatous polyposis coli
ARPC1A	actin related protein complex, subunit 1A
ATR	ataxia telangiectasia and Rad3 related
BAC	bacterial artificial chromosome
BAX	BCL2-associated X protein
BCR	breakpoint cluster region
BER	base excision repair
BFB	breakage-fusion-bridge
BCL2	B-cell leukemia/lymphoma 2
BMI-1	B lymphoma Mo-MLV insertion region 1 homolog
BRCA2	breast cancer 2
CLL	chronic lymphocytic leukemia
CCNA2	cyclin A2
CDC16	cell division cycle 16 homolog
CDK	cyclin-dependent kinase
CDKN	cyclin-dependent kinase inhibitor
cDNA	complementaryDNA
CGH	comparative genomic hybridization
CSC	cancer stem cell
CT	computed tomography
CTD	C-terminal domain
DM	couble minute
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DPC4	deleted in pancreatic cancer 4
DYRK1B	dual-specificity tyrosine phosphorylation regulated kinase 1B
EGFR	epidermal growth factor receptor
EID2B	EP300 interacting inhibitor of differentiation 2B
ERCP	endoscopic retrograde cholangiopancreatography
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
ETS	the E twenty-six
FAMMM	familial atypical mole-multiple melanoma

FAP	familial adenomatous polyposis
FISH	fluorescence *in situ* hybridization
GADD45	growth arrest and DNA-damage-inducible 45 alpha
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GMFG	glia maturation factor gamma
GTF2H1	general transcription factor IIH, polypeptide 1
GTSE1	G-2 and S-phase expressed 1
GUSB	glucuronidase beta
HAT	histone acetyltransferase
HDA	histone deacetylase
HERC5	hect domain and RLD 5
HNPCC	hereditary nonpolyposis colorectal cancer
HSR	homogenously staining region
HT-RNAi	high-throughput RNAi
IXL	intersex-like
IPMN	intraductal papillary mucinous neoplasm
JNK	c-Jun N-terminal kinase
kb	kilobase
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LRFN1	leucine-rich fibronectin 1
LUC	luciferase
MANM	multiple allosteric networks model
MAPK	mitogen activated protein kinase
Mb	megabase
MCN	mucinous cystic neoplasms
MED29	mediator subunit 29
MIF	macrophage migration inhibitory factor
miRNA	micro-RNA
MLH1	mutL homolog 1
MMR	mismatch repair
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSH2	mutS homolog 2
MYB	v-myb myeloblastosis viral oncogene
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NER	nucleotide excision repair
PAF1	RNA polymerase II associated factor
PAK4	p21 activated kinase 4
PanIN	pancreatic intraepithelial neoplasia
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PDGF	platelet derived growth factor
PIC	preinitiation complex
PLEKHG2	pleckstrin homology domain containing family G member 2
Pol	polymerase
PSMC4	proteasome 26S ubunit ATPase 4
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real-time PCR
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1

RB1	retinoblastoma 1
RBBP8	retinoblastoma binding protein 8
RE	response element
ROMA	representational oligonucleotide microarray analysis
RNA	ribonucleic acid
RNAi	RNA inhibition
SH3	Src homology 3
SHH	Sonic hedgehog
siRNA	small inhibitory RNA
SAMD4B	sterile alpha motif domain containing 4B
SMAD	mothers against decapentaplegic homolog 4
SMURF1	Smad ubiquitination regulatory factor 1
SNP	single nucleotide polymorphism
SRE	serum response element
STK11	serine/threonine kinase 11
SUMO1	SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae)
SUPT5H	suppressor of Ty 5 homolog (S. cerevisiae)
TBP	TATA-box binding protein
TF	transcription factor
TGF-β	transforming growth factor beta
TMA	tissue microarray
TMPRSS2	transmembrane protease, serine 2
TP53	tumor protein p53
UAS	upstream activated sequence

ABSTRACT

Pancreatic cancer is one of the most aggressive malignancies with high mortality. In Finland, it affects about approximately 900 people annually and nearly the same number of people die of it. The current treatment strategies for pancreatic cancer are ineffective, and the prognosis is very poor. Ductal adenocarcinoma is the most common form of pancreatic cancer and the term 'pancreatic cancer' refers here to the ductal subtype. This study aimed to characterize the molecular aberrations in pancreatic cancer and to highlight putative target genes that may serve as novel diagnostic markers or targets for therapy. More specifically, it concentrated on the characterization of the amplified region at chromosome 19q13 and functional studies of putative target genes within this locus.

The first study explored DNA copy number changes at the 19q13 locus in pancreatic cancer cell lines using fluorescence *in situ* hybridization (FISH). A 1.1 Mb amplicon with a 660-kb core region of high-level amplification was delineated. The prevalence of the 19q13 amplification and its possible correlation with clinicopathological features was investigated in over 500 primary pancreatic tumors. 19q13 copy number changes were found in more than 10% of ductal adenocarcinomas. Importantly, these changes correlated with advanced tumor stage and grade. In addition, the median survival time of patients with 19q13 amplification was considerably shorter than that of patients with a normal copy number.

Genes located within the amplified region were identified using public genomic databases, and their expression levels were defined by quantitative real-time PCR (qRT-PCR). Interestingly, a number of genes within the amplicon were overexpressed through amplification. To select functionally important targets, an RNA inhibition (RNAi)-based viability screen was applied to identify genes for which downregulation attenuated cell viability. The expression screen and RNAi profiling results identified *MED29* (*IXL*) as the most promising candidate target in the 19q13 amplicon.

Subsequent functional studies concentrated on MED29. RNAi-mediated downregulation of MED29 in cells with high endogenous MED29 expression led to inhibition of several cancer cell-associated characteristics, such as cell growth, migration, invasion, and colony formation on soft agar. Unexpectedly, the lentiviral-based induction of MED29 expression in pancreatic cancer cells with low endogenous MED29 levels also led to growth inhibition. More important, when MED29-overexpressing pancreatic cancer cells were inoculated into nude mice, a dramatic reduction in tumor incidence and tumor growth was observed compared to mice inoculated with control cells.

A genome-wide microarray-based gene expression analysis was utilized to uncover the mechanisms involved in the growth differences between the MED29 and the control cells. This analysis identified a large group of differentially expressed genes, and subsequent ontology analyses revealed their association with cell cycle- and cell division-related processes. Overall, the microarray data show that several cell cycle regulatory genes were affected by MED29 expression in a way that led to cell cycle inhibition.

To conclude, amplification of the 19q13 chromosomal locus has clinical significance and correlates with tumor stage and grade in pancreatic cancer. Amplification of 19q13 leads to the upregulation of multiple genes, of which *MED29* represents a putative target gene. Silencing of *MED29* in pancreatic cancer cells with a high MED29 expression level resulted in reduced cell growth and survival and inhibited several cancer cell-associated characteristics. In contrast, induced MED29 overexpression in pancreatic cancer cells with low endogenous levels of the protein led to reduced growth and, importantly, inhibited tumor formation in a mouse xenograft model. MED29 is part of a large, multisubunit complex that is involved in the regulation of transcription and has recently been linked to the pathogenesis of several cancers. This study shows for the first time that MED29 possesses an important but complex role in pancreatic cancer pathogenesis.

INTRODUCTION

The pancreas is a glandular organ located in the abdominal cavity behind the stomach. It is an important regulator of protein and carbohydrate digestion and glucose homeostasis. The exocrine component of the pancreas is composed of acinar and ductal cells that produce and deliver pancreatic juice containing digestive enzymes into the gastrointestinal tract to facilitate the breakdown of carbohydrates, proteins and fats (Bardeesy & DePinho, 2002; Hezel et al., 2006). The endocrine component of the pancreas is composed of four specialized cell types that form clusters called Islets of Langerhans. It is responsible for the regulation of metabolism and glucose homeostasis through the secretion of hormones, including insulin, glucagon and somatostatin (Bardeesy & DePinho, 2002; Hezel et al., 2006). Typical diseases of the pancreas include diabetes, pancreatitis and pancreatic cancer.

Pancreatic cancer affects approximately 230,000 people every year worldwide and is the eighth most common cause of cancer-related death (Parkin et al., 2005). In Finland, pancreatic cancer accounts for approximately 900 cases per year and is slightly more common among men than women (The Finnish Cancer Registry). The median survival of patients with pancreatic cancer is less than six months, and the five-year survival rate is less than 5% (Hezel et al., 2006; Jemal et al., 2009). The poor prognosis of this malignancy results from its ability to rapidly invade the lymphatic system and surrounding tissues and to metastasize to distant sites before diagnosis (Hezel et al., 2006). The aggressive biology of pancreatic cancer and its resistance to available therapies generally preclude curative treatment at the time of diagnosis. Less than 20% of pancreatic carcinomas are localized to the pancreas and are resectable when diagnosed (Freitas et al., 2009; Hidalgo, 2010).

Pancreatic cancer evolves through sequential genetic and epigenetic changes in the ductal epithelium over a prolonged period (Bardeesy & DePinho, 2002; Maitra & Hruban, 2008). Pancreatic cancer seldom affects young people; it is a disease of the elderly caused by the gradual accumulation of genetic and epigenetic changes that eventually provide tumor cells with a growth advantage and positive selection

over non-cancerous cells. The histopathological picture of pancreatic carcinoma and its precursor lesions, together with the accompanying molecular profiles, have formed the framework for current research on pancreatic cancer (Hruban et al., 2000; Bardeesy & DePinho, 2002). In addition, recent methodological achievements as well as the sequencing of the human genome have revolutionized cancer research and provided excellent tools for the exploration of the molecular events that take place in the development of cancer (Bell, 2010). High-throughput profiling technologies have produced a wealth of data pertaining to somatic alterations in pancreatic cancer, including mutations, deletions, and amplifications (Jones et al., 2008), thus increasing our knowledge of the molecular events that take place in pancreatic cancer tumorigenesis. However, a number of cancer-associated genes remain to be discovered and functionally characterized within the altered regions (Buchholz & Gress, 2009).

This study aims to investigate specific molecular alterations in pancreatic cancer and explore their clinical importance, identify putative new amplification target genes, and finally, characterize the function of the most promising candidates.

REVIEW OF THE LITERATURE

1. Molecular alterations in cancer

Cancer is thought to evolve through the accumulation of genetic and epigenetic changes that confer cancerous cells a selective advantage over the non-cancerous cell population (reviewed by Ponder, 2001). It is seen as a stepwise evolutionary process where a single mutation provides an initial growth or survival advantage to one cell and is then followed by additional changes, finally leading to the clonal expansion of cells with multiple genetic changes and unlimited growth potential (reviewed by Kinzler & Vogelstein, 1996). During tumor development, a cancer cell must acquire certain characteristics to become malignant: self-sufficiency in growth signals, insensitivity to growth regulatory signals, a means to escape from apoptosis, unlimited replication potential, sustained angiogenesis, and the ability to invade tissue and metastasize, the features generally known as the hallmarks of cancer (reviewed by Hanahan & Weinberg, 2000).

Cancer cells obtain these characteristics through diverse mechanisms that lead to changes in gene sequence, structure, copy number, and expression (Albertson, 2006; Bell, 2010). These changes can affect the target protein in a way that allows tumor cells a growth advantage or prolonged survival. Aberrations are brought about at the genomic level by various mechanisms, including altered karyotypes, mutations and epigenetic regulation (Bell, 2010). Mutations, defined as permanent changes in the DNA sequence, are generally divided into small and large-scale aberrations. Small-scale mutations include small deletions, insertions, and point mutations that can lead to a truncated, altered, or unaffected protein product (Vogelstein & Kinzler, 2004). A point mutation causes a single nucleotide exchange that can be silent (does not change the amino acid), cause a change in the amino acid sequence and possibly altered protein structure (missense mutation) or introduce a premature stop codon and result in a truncated protein (nonsense mutation) (Vogelstein & Kinzler, 2004). Deletions and insertions typically result in frameshift

changes (i.e., a shift in the reading frame of the nucleotide codon during translation) that usually lead to an altered amino acid sequence and changed protein. They can also result in splice site mutations if the insertion or deletion of nucleotides occurs at the site of splicing (removement of introns) during the RNA processing. Large-scale mutations or chromosome-level alterations involve gains and losses of chromosomal loci (i.e., amplifications and deletions) and chromosomal rearrangements, such as inversions and translocations (Albertson et al., 2003). Amplifications (see detailed discussion in the following chapter) and deletions cause changes in gene dosage and are commonly observed in human cancers. Inversion is a chromosomal rearrangement in which a segment of a chromosome is reversed within a single chromosome. Translocation involves the rearrangement of two chromosomes. Translocations may lead to the formation of chimeric proteins when two genes are joined together (Croce, 2008). Genes can also be translocated to sites at which their transcription is affected. Chromosomal abnormalities also include aberrations in chromosome number, referred to as aneuploidy, which are characteristic of several human cancers.

1.1 Tumor suppressor genes and oncogenes

Acquired or inherited aberrations typically affect two major classes of cancer genes: tumor suppressor genes and oncogenes (reviewed by Vogelstein & Kinzler, 2004). Tumor suppressor genes can be divided into three classes: gatekeepers, caretakers, and landscapers. Gatekeepers are growth regulatory genes that control important cellular processes such as proliferation, cell cycle and DNA repair (Sherr, 2004; Stratton, 2009). They usually become inactivated during tumor development through allelic loss, an inactivating mutation or epigenetic silencing (Vogelstein & Kinzler, 2004). These inactivating events are generally referred to as loss-of-function aberrations. According to the Knudson two-hit hypothesis (Knudson, 1971), two hits are needed to inactivate both alleles of a tumor suppressor gene. If only one allele is affected, the remaining allele can produce a functional protein. However, in the case of haploinsufficiency, the loss of a single allele is sufficient to abolish the function of the gene (Fero et al., 1998). The first identified tumor suppressor gene was *RB1* (retinoblastoma 1), which is recognized as a causative

affected gene in retinoblastoma patients (Knudson, 1971). Another well-known example of a tumor suppressor gene that is commonly silenced during tumor development is *TP53* (Sherr, 2004). *TP53* encodes the p53 protein, which plays a fundamental role in the control of the cell cycle and the induction of apoptosis. Another class of tumor suppressor genes includes the caretakers that sustain genomic integrity and thus indirectly control cancer progression by maintaining a low mutation rate (Kinzler & Vogelstein, 1997). This subclass of tumor suppressors includes mismatch repair (MMR) genes, nucleotide-excision repair (NER) genes, and base-excision repair (BER) genes, which are all responsible for correcting mistakes that arise in DNA during replication (Vogelstein & Kinzler, 2004). Some stability genes, for example, *BRCA1* and *BRCA2*, control large genome-related processes such as recombination and chromosomal segregation (Vogelstein & Kinzler, 2004). Mutations in caretakers commonly lead to genomic instability. The third subclass of tumor suppressor genes, called landscapers, includes genes that modulate the microenvironment in which the cells grow (Kinzler & Vogelstein, 1998). The growth of tumor cells is highly dependent on their interactions with stromal cells and the extracellular matrix (Bissell & Radisky, 2001). Faulty landscaper genes could contribute to the growth of tumor cells by causing abnormalities in the tumor microenvironment (Macleod, 2000). *PTEN* and *SMAD4* have been indicated as potential landscaper genes (Kinzler & Vogelstein, 1998; Macleod, 2000).

Proto-oncogenes are normal cellular genes that can turn into oncogenes when abnormally activated, most commonly by mutation, amplification, or chromosomal rearrangement (Figure 1). Accordingly, alterations leading to the activation of an oncogene are called gain-of-function mutations. They cause changes in the DNA that affect the protein product in a way that leads to abnormal activity or loss of regulation, thus providing the tumor cell with a growth advantage (Todd & Wong, 1999; Vogelstein & Kinzler, 2004). A point mutation in a proto-oncogene can change the protein structure in a way that alters its normal function, such as by constitutive activation. Translocations, which are caused by chromosomal rearrangements, may give rise to novel fusion proteins with altered function and capability. A classical example of such an oncogenic fusion gene is *ABL-BCR* generated by a t(9;22) translocation. The resulting chromosome is known as the Philadelphia chromosome in chronic myelogenous leukemia (Shtivelman et al.,

1985). Alternatively, a gene can be translocated to a new location that is under the control of a strong promoter, leading to induced expression. Translocations are more common in lymphomas and leukemias, but they are also found in solid tumors (Nambiar et al., 2008). As an example, fusion of the *TMPRSS2* gene with *ETS* transcription factor genes has been observed in prostate cancer (Tomlins et al., 2005). Amplification refers to a selective increase in gene dosage, which often leads to increased messenger RNA (mRNA) and protein expression (Albertson, 2006). It is a typical mechanism of oncogene activation and will be discussed later in more detail (see below). In contrast to tumor suppressor genes, oncogenes act in a dominant fashion, meaning that a single altered allele provides a tumor cell with a growth advantage and may lead to uncontrolled growth (Todd & Wong, 1999). Oncogenes typically encode growth factors (e.g., *PDGF*), growth factor receptors (e.g., *ERBB2*), transcription factors (e.g., *MYC*), chromatin remodelers (e.g., *ALL1*), signal transducers (e.g., *RAF1*), and apoptosis regulators (e.g., *BCL2*) (Croce, 2008).

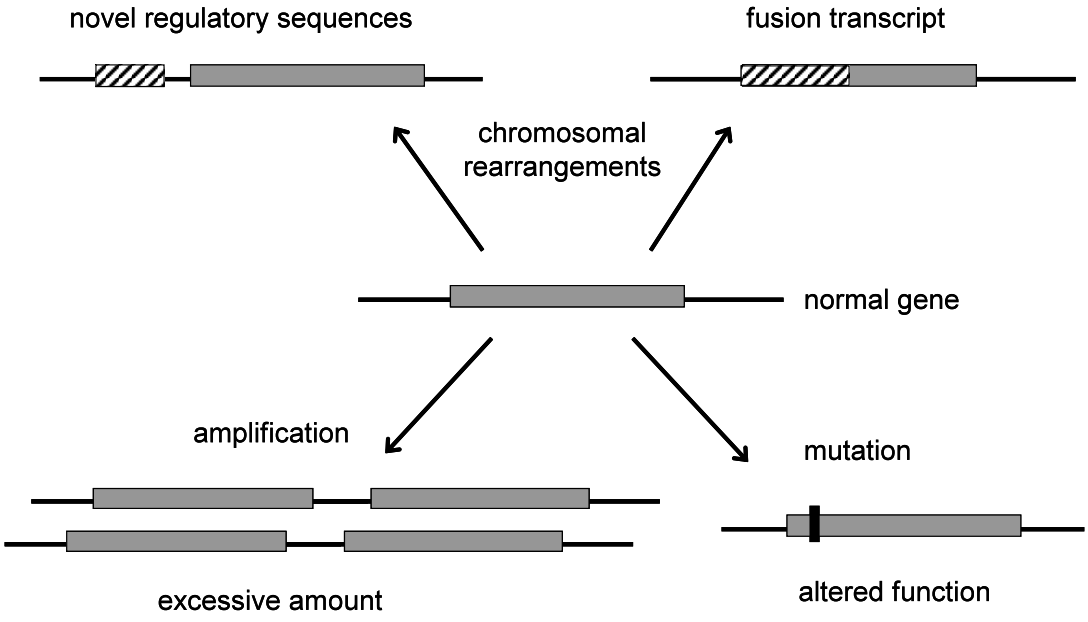

Figure 1. *Common oncogene activation mechanisms.*

1.2 Gene amplification in cancer

Gene amplification, defined as a selective increase of DNA copy number in a specific chromosomal region, is a common mechanism used by solid tumors to activate genes that contribute to tumor development (Albertson et al, 2003). Increased DNA copy number of an otherwise normal gene is expected to lead to increased levels of mRNA and protein, thus giving rise to increased activity or function of the protein.

1.2.1 Manifestation and mechanisms

Amplified DNA can be organized in different ways in mammalian nuclei: as extrachromosomal units, consisting of sub-microscopic episomes and cytogenetically visible double minutes (DMs); as repeated elements at a single locus, seen as homogenously staining regions (HSRs); or scattered at various sites throughout the genome (Schwab, 1999; Albertson, 2003). HSRs, DMs, and episomes may contain genetic material from different chromosomal origins (Myllykangas & Knuutila, 2006). DMs are acentric, atelomeric, and autonomously replicating circular segments of DNA (Hahn, 1993; Albertson, 2006) that are thought to arise via episome excision (Wahl et al., 1989). They can also be formed through HSR breakdown and subsequent circularization (Singer et al., 2000). Similarly, DMs can relocate into the genome after a double-stranded DNA break to form an HSR (Coquelle et al., 1998). According to the breakage-fusion-bridge (BFB) model, which was originally proposed by McClintock based on her studies of maize, amplification evolves from repeated cycles of fusion and breakage of chromosomes (McClintock, 1942). This cycle begins with the formation of a dicentric chromosome resulting from a chromosomal break or telomere dysfunction. At anaphase, the centromeres are pulled in opposite directions and form a bridge. A breakage generates sticky ends that fuse after replication, generating a chromosome with inverted duplicated terminal sequences (Savelyeva & Schwab, 2001). This cycle goes on until the chromosomal ends are stabilized. The sequential events in the BFB cycle, including amplification due to telomere loss (Murnane & Sabatier, 2004) or chromosomal breakage (Coquelle et al., 1997), formation of fused sister chromatids and anaphase bridges (Shimizu et al., 2005), formation of inverted

repeats (Toledo et al., 1992), and intra-tumor heterogeneity (Gisselsson et al., 2000), have been observed *in vivo*, providing further support for this model. Additional amplification mechanisms have been proposed, including over-replication and unequal exchange (Windle & Wahl., 1992; Smith, 1990). The over-replication model is based on multiple initiations of replication during a single cell cycle, giving rise to onion skin-like structures (Windle & Wahl, 1992), whereas unequal exchange is based on unequal recombination of either homologous or non-homologous chromatids, leading to duplication in one chromatid and deletion in the other (Smith, 1990). Common chromosomal fragile sites, defects in DNA replication and telomere dysfunction are thought to promote amplification events (Myllykangas & Knuutila, 2006).

1.2.2 Clinical implications

Gene amplification in mammals is an unscheduled event that occurs in response to cytotoxic drugs and during tumorigenesis (Schwab, 1999). It has been associated with aggressive tumors and decreased survival (Savelyeva & Schwab, 2001). Therefore, it may be useful as a diagnostic and prognostic marker in the prediction of therapeutic response and as a target for drug design (Albertson et al., 2003). Amplified targets with clinical significance as prognostic markers include the *MYCN* oncogene in neuroblastoma (Savelyeva & Schwab, 2001) and *MYC* and *ERBB2* in breast cancer (Slamon et al., 1987; Al-Kuraya et al., 2004). Amplification of the *ERBB2* oncogene is assessed to predict the therapeutic response and select appropriate treatment strategies for breast cancer (Masood et al., 2002; Mass et al., 2005). In addition, amplified oncogenes can be excellent targets for specific therapies because tumors can become dependent on their increased expression, a phenomenon known as “oncogene addiction” (Weistein et al., 2002). A well-known example of an anti-cancer agent targeting a specific amplified oncogene is trastuzumab (Herceptin), which targets *ERBB2* (Carter et al, 1992).

1.2.3 Target identification

Amplifications and other copy number variations (gains and losses) can be detected at the genomic level by several methods, most commonly by microarray-based approaches. The development of new microarray technologies and high-throughput screening methods has boosted the mapping of tumor genomes and eased the identification of tumor-associated genes. Conventional comparative genomic hybridization (CGH) (Kallioniemi et al., 1992), which is based on competitive hybridization of tumor and reference DNA to metaphase chromosomes, has provided the basis for the methods currently used for the detection of unbalanced variants in tumor genomes. CGH can be applied to obtain copy number data at the chromosome band-level, whereas spotted arrays provide much better resolution. CGH arrays with different platforms, such as BAC clones (Cai et al., 2002), cDNA clones (Pollack et al., 1999), and oligonucleotides (Barrett et al., 2004), have been developed. In addition, microarrays designed for single nucleotide polymorphism (SNP) detection can be applied to detect DNA copy number changes.

It is necessary to confirm the results of an array-CGH (aCGH) experiment once a copy number increase is found. Confirmation is usually achieved by fluorescence *in situ* hybridization (FISH) or quantitative PCR (qPCR). FISH can also be applied to further resolve amplification profiles and to redefine the boundaries of an amplicon (Feuk et al., 2006). Briefly, molecularly cloned genomic DNA sequences (typically BAC clones) are chosen to cover the region of interest, followed by fluorescent labeling and hybridization to interphase chromosomes of the investigated material. Detection of copy number signals can be laborious by this approach, but it can be applied to tissue samples and utilized at a large scale if the sample material is printed on a single slide, for example, tissue microarrays (TMAs) (Kononen et al., 1998).

Delineation of the amplicon is followed by the identification of genes within the amplified region and expression analysis by quantitative RT-PCR. Expression analysis is performed because amplification target genes are expected to be overexpressed (Albertson, 2006). Identification of the so-called driver genes (genes that promote tumor development) for specific amplicons remains challenging because several genes can be amplified and overexpressed within a single amplicon (Bärlund et al., 2000; Kauraniemi et al., 2001; Hyman et al., 2002; Heidenblad et al,

2005; Pärssinen et al., 2007). Amplicons often contain so-called passenger genes that are amplified and overexpressed along with the driver genes but do not promote tumor formation. Therefore, functional validation of putative targets is required. Efficient methods to obtain biological confirmation of the causal role of putative targets include the RNA interference (RNAi)-mediated knockdown of amplified genes in overexpressing cells and ectopic expression of target genes in a model lacking the amplification (Santarius et al., 2010). At this time, 77 amplified human genes with sufficient evidence for a causal role in cancer have been identified (Santarius et al., 2010).

1.2.4 The 19q13 amplicon

A genome-wide aCGH profiling (Mahlamäki et al., 2004) revealed a high-level amplification at 19q13 in pancreatic cancer, which was subsequently confirmed by other aCGH studies (Aguirre et al., 2004; Heidenblad et al., 2004; Holzmann et al., 2004; Bashyam et al., 2005; Gysin et al., 2005, Nowak et al., 2005). The high-amplification peak in the aCGH profile suggests that this amplicon might be biologically important. During the past few years, the 19q13 amplicon has gained attention in pancreatic cancer, and several putative target genes have been reported, including *DYRK1B* (Moniaux et al., 2006), *PAF1* (Deng et al., 2006) and *PAK4* (Chen et al., 2008). *AKT2*, a putative oncogene in ovarian cancer, encodes for a serine/threonine protein kinase and is also located within this amplicon (Cheng et al., 1992).

In addition to pancreatic carcinomas, 19q13 amplification has been observed in a subset of other cancers, including ovarian cancer (Cheng et al., 1992; Bellacosa et al., 1995; Thompson et al., 1996; Tang et al., 2002), breast cancer (Kallioniemi et al., 1994; Bellacosa et al., 1995; Yu et al., 2009), cervical cancer (Rao et al., 2004; Narayan et al., 2007), gastric cancer (Vauhkonen et al., 2007), non-small cell lung cancer (Kim et al., 2005), small cell lung cancer (Ried et al., 1994; Petersen et al., 1997), and urinary bladder cancer (Richter et al., 2000). Table 1 provides examples of studies showing 19q13 amplification in several cancers. Only relatively common cancers with adequate sample size and a mention of the 19q13 amplification are included in Table 1. The 19q13 aberration is a fairly rare event, but it often

manifests as a high-level amplification (over 10 copies per cell), suggesting biological significance. Importantly, the exact size and location of the amplifications in different studies may vary due to the fact that different methods have been used. Therefore, one cannot be certain that these amplicons represent the same locus. It is possible that several regions of amplification may exist within the 19q13 locus.

Table 1. *Examples of studies showing 19q13 amplification in various tumors.*

Reference	Tumor type	No. of samples	Method
Dohna et al., 2000	Adenocortical carcinoma	22 primary tumors	CGH
Kallioniemi et al., 1994	Breast cancer	33 carcinomas	CGH
Bellacosa et al., 1995	Breast cancer	106 carcinomas	Southern
Yu et al., 2009	Breast cancer	16 carcinomas	aCGH
Rao et al., 2004	Cervical cancer	77 carcinomas	CGH
Narayan et al., 2007	Cervical cancer	21 primary tumors, 8 cell lines	aCGH
Larramendy et al., 1997	Chondrosarcoma	19 tumors	CGH
Knösel et al., 2004	Colorectal cancer	54 carcinomas	CGH
Sniders et al., 2003	Fallopian tube cancer	14 carcinomas	aCGH
van Dekken et al., 2001	Gastric cancer	20 carcinomas	aCGH
Vauhkonen et al., 2007	Gastric cancer	15 carcinomas	aCGH
Huntsman et al., 1999	Glioblastoma	5 cell lines	FISH
Marchio et al., 1997	Hepatocellular cancer	50 carcinomas	CGH
Medina et al., 2009	Lung cancer	25 primary tumors	aCGH, FISH
Terris et al., 1998	Neuroendocrine tumors of digestive system	20 tumors	CGH
Balsara et al., 1997	Non-small cell lung cancer	10 carcinomas, 10 cell lines	CGH
Kim et al., 2005	Non-small cell lung cancer	50 carcinomas	aCGH
Atiye et al., 2005	Osteosarcoma	22 sarcomas	aCGH
Bellacosa et al., 1995	Ovarian cancer	132 carcinomas	Southern
Yamamoto et al., 2009	Ovarian cancer	136 carcinomas	FISH
Mahlamäki et al., 2004	Pancreatic cancer	16 cell lines	aCGH
Kikuchi et al., 2008	Pancreatic cancer	29 carcinomas	FISH
Chen et al., 2008	Pancreatic cancer	42 carcinomas, 27 cell lines	ROMA
Ganguly et al., 2009	Retinoblastoma	25 blastomas	SNP genotyping
Ried et al., 1994	Small cell lung cancer	13 primary tumors	aCGH
Richter et al., 2000	Urinary bladder cancer	1561 carcinomas	FISH

aCGH, array comparative genomic hybridization; CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; ROMA, representational oligonucleotide microarray analysis; SNP, single nucleotide polymorphism

1.3 Epigenetics

In addition to genetic alterations, it has become apparent that epigenetic mechanisms play an important role in the evolution and progression of cancer (Omura & Goggins, 2009; Feinberg et al., 2006; Ting et al., 2006; Jones & Baylin, 2007; Esteller, 2007). Epigenetics is defined as hereditable changes in gene expression that are not caused by changes in DNA sequence. These events are typically mediated by two mechanisms: DNA methylation and histone modification.

DNA methylation involves the covalent addition of a methyl group to the 5' position of cytosine pyrimidine, most often at CpG dinucleotides (Omura & Goggins, 2009). This modification is brought about and maintained by DNA methyltransferases (DNMTs) (Yen et al., 1992; Okano et al., 1998). DNA methylation is crucial for normal mammalian development (Okano et a, 1999) but is also a key regulator of gene expression in various cancers (Omura & Goggins, 2009). Several tumor suppressor genes are silenced through promoter hypermethylation in cancer but even hypomethylation has been observed in the context of gene overexpression (Sato et al., 2003a).

Histones are the backbone proteins of chromatin, which package DNA into structural units called nucleosomes. A nucleosome is a hetero-octamer composed of core histone proteins and tightly wound DNA around it. Long, protruding N-terminal tails of histones on the surface of nucleosomes serve as the main sites of biochemical modification, including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP-ribosylation (Esteller, 2007; Kouzarides, 2007). Acetylation of lysine residues by histone acetyl transferases (HATs) neutralizes the charge between the histone tails and the negatively charged DNA, making chromatin more accessible for active transcription. Correspondingly, histone deacetylation is mediated by another group of specific enzymes called histone deacetylases (HDAs). Histone deacetylation serves as an alternative and combinatory route for epigenetic silencing of tumor suppressor genes.

1.4 Micro-RNAs

Micro-RNAs (miRNAs) are single-stranded, non-coding RNA molecules of ~22 nucleotides that function as negative regulators of gene expression in various biological processes, such as proliferation, differentiation, and apoptosis (reviewed by Esquela-Kerscher & Slack, 2006). Most human miRNAs are located within introns of protein-coding or non-coding mRNAs and are transcribed by RNA polymerase II. MiRNAs are often only partially complementary to their target mRNA sequence and can exert silencing either by translational repression or mRNA degradation, depending on the degree of complementarity (Elbashir et al., 2001; Brennecke et al., 2005). Perfect or nearly perfect complementarity to target mRNA

induces RNA interference (RNAi), leading to the complete degradation of target mRNA, whereas imperfect complementarity leads to translational repression. A single miRNA can control hundreds of target genes (Lim et al, 2005; Barbato et al., 2009) and approximately 30% of human genes are estimated to be targeted by miRNAs (Lewis et al., 2005; Xie et al., 2005). Aberrant miRNA expression in cancer was first observed a few years ago as two miRNAs, miR-15a and miR-16-1, were shown to be downregulated or deleted in chronic lymphocytic leukemia (CLL) (Calin et al, 2002). Subsequently, several miRNA profiles of various cancers have shown aberrant regulation and expression of multiple miRNAs (Calin et al, 2006; Zhang et al., 2006; Lee et al., 2007; Porkka et al., 2007). Moreover, recent miRNA expression profiling containing multiple cancers clustered different tumor types more accurately than mRNA expression profiling (Lu et al., 2005). MiRNAs are thought to function in cancer as tumor suppressor genes and oncogenes (Esquela-Kersher & Slack, 2006).

2. Pancreatic cancer

2.1. Epidemiology and risk factors

Pancreatic cancer is an aggressive disease and one of the deadliest malignancies. In 2007, age-adjusted incidence rates in Finland were 9.3 per 100,000 among men and 6.6 per 100,000 among women, whereas mortality rates climbed to 9.0 and 6.9 per 100,000, respectively (Finnish Cancer Registry, http://www.cancerregistry.fi). Late diagnosis and ineffective treatment strategies make pancreatic cancer the third most common cause of cancer-related death in Finland. These rates are similar to those observed in the Western world (Finnish Cancer Registry, Jemal et al., 2009; Thun et al., 2010). Prognosis in pancreatic cancer is poor because of the aggressiveness of the disease, lack of early diagnostic tools and profound resistance to treatment. Median survival for patients with locally advanced disease is somewhat better than for those with metastatic disease, but the overall five-year survival rate among pancreatic cancer patients is less than 5% (Jemal et al., 2009; Thun et al., 2010).

The causes of pancreatic cancer have been widely explored, but they remain largely unknown (reviewed by Hidalgo, 2010). Only a few demographic and environmental risk factors and some autosomal dominant genetic disorders have been linked to pancreatic cancer (Hezel et al., 2006, Raimondi, 2009). Involvement of various environmental risk factors has been implicated, but clear evidence of a causative role exists only for smoking (Fuchs et al., 1996; Schuller, 2002; Hezel et al., 2006; Raimondi, 2009). Smoking doubles the risk of pancreatic cancer (Maitra & Hruban, 2008) and approximately 25% of pancreatic cancer cases are related to smoking (Raimondi, 2009). The association between pancreatic cancer and dietary factors has been continuously investigated, but no strong or consistent correlation has been confirmed (Raimondi, 2009). Obesity and a diet high in meats and fat are reported to increase the risk of pancreatic cancer (Michaud et al., 2001; Larsson et al., 2007), whereas a so-called healthy lifestyle appears to decrease the risk (Jiao et al., 2009). Alcohol consumption does not appear to be a causal factor for pancreatic cancer unless it leads to chronic pancreatitis, which has been linked to an increased risk (Lowenfels et al., 1993; Lowenfels & Maisonneuve, 2005; Maitra & Hruban, 2008; Raimondi, 2009). Other known risk factors include advanced age, a family history of pancreatic cancer (Klein et al., 2004), and a hereditary predisposition (discussed below). Long-standing diabetes confers an increased risk of developing pancreatic cancer, but it can also be an early manifestation of the disease (Everhart & Wright, 1995; Huxley et al., 2005).

Approximately 10% of pancreatic cancer patients have an underlying germline disorder, while the rest of the cases result from somatic changes (Lynch et al., 1996; Schenk et al., 2001; Hezel et al., 2006; Lochan et al., 2008). Hereditary diseases and cancer syndromes associated with pancreatic cancer include familial pancreatitis (Lowenfels et al., 1997), familial breast and ovarian cancer (Goggins et al; 1996; Lynch et al., 2005), familial atypical multiple mole melanoma (FAMMM) (Bartsch et al., 2002; Goldstein et al., 2004), Peutz-Jeghers syndrome (Giardiello et al., 2000), Li-Fraumeni syndrome (Kleihues et al., 1997), hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (Yamamoto et al., 2001; Geary et al., 2008), familial adenomatous polyposis (FAP) (Giardiello et al., 1993), and cystic fibrosis (Maisonneuve et al., 2007). Germline mutations associated with an increased risk for pancreatic cancer include among others those targeting the tumor suppressor genes *APC*, *BRCA2*, *CDKN2A/p16*, and *STK11/LKB1*, the DNA mismatch repair

gene *MLH1*, and the trypsinogen gene *PRSS1* (Hezel et al., 2006). Germline mutations in *CDKN2A* are primarily linked to FAMMM, but they confer up to a 22-fold increased risk for pancreatic cancer (Goldstein et al, 2004). *BRCA1* and *BRCA2* mutations are responsible for familial breast and ovarian cancers, but they are also associated with increased susceptibility to pancreatic cancer. *BRCA1* is associated with a lower relative risk than *BRCA2* (Goggins et al; 1996; Lynch et al., 2005). Silencing of *STK11/LKB1* is characteristic of Peutz-Jeghers syndrome and confers a substantially increased risk of developing pancreatic cancer. Inherited mutations in DNA mismatch repair genes, including *MLH1* and *MSH2,* are typically found in HNPCC, but they are also linked to pancreatic cancer susceptibility (Yamamoto et al., 2001; Geary et al., 2008). Further, patients with familial pancreatitis caused by germline mutations in *PRSS1* have an over 50-fold increase in the incidence of pancreatic cancer (Lowenfels et al., 1997). FAP is an autosomal dominant disease of the colon caused by mutated *APC*, which confers an intermediate risk for pancreatic cancer (Giardiello et al., 1993). The germline mutations discussed here account for less than 20% of pancreatic cancer prone familial cases, implying that additional predisposing genes and loci exist (Hezel at el., 2006). One such susceptibility locus showing autosomal dominant inheritance has been identified at the 4q32-43 locus (Eberle et al., 2002).

2.2. Pancreatic cancer pathology

The pancreas is composed of two compartments: the exocrine and the endocrine pancreas. The endocrine pancreas contains four special cell types gathered together into clusters called Islets of Langerhans that are embedded between the exocrine units of the pancreas (Figure 2) (Bardeesy & DePinho, 2002; Hezel et al., 2006). The endocrine pancreas regulates glucose homeostasis through the secretion of hormones such as insulin and glucagon. The exocrine compartment of the pancreas accounts for 80% of the tissue mass and is composed of three types of cells: ductal, acinar and centroacinar cells (Hezel et al., 2006). Ductal cells are cubically shaped cells that form the ductal network of the pancreas. The acinar cells are organized into functional units at the end of the ducts (Figure 2). The centroacinar cells are located in the acinar units between the acinar and the ductal cells (Hezel et al.,

2006). The exocrine pancreas is involved in digestion and secretes digestive enzymes into the duodenum.

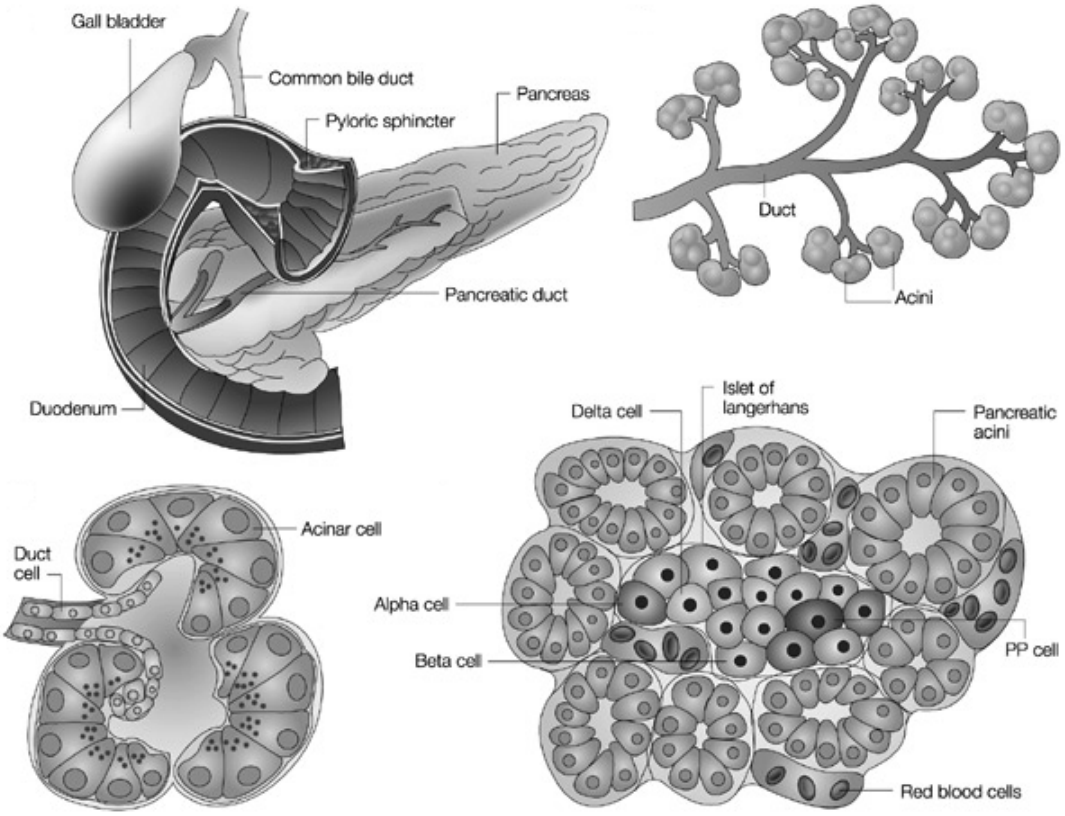

Figure 2. *Anatomy of the pancreas illustrating its gross anatomy (upper left), the exocrine compartment (upper right), a single acinus (lower left) and a pancreatic islet surrounded by the exocrine tissue (lower right) (Bardeesy & DePinho, 2002. Reprinted with permission).*

Tumors of the pancreas are roughly divided into endocrine and exocrine tumors. This study focuses on exocrine tumors, and more specifically, on pancreatic ductal adenocarcinoma (PDAC), which is the most common form of pancreatic cancer, accounting for approximately 85% of all cases (Mihaljevic et al., 2010). According to the WHO (World Health Organization) classification system, pancreatic exocrine tumors are divided into benign, borderline, and malign tumors according to their biological behavior. They are histologically graded as well, moderately, and poorly differentiated (Klöppel et al., 1996). The most widely recognized staging of pancreatic cancer relies on TNM classification from 1987, which is based on the extent of the primary tumor (T), regional lymph node status

(N), and metastatic status (M). Pancreatic exocrine carcinomas encompass ductal adenocarcinomas, osteoclast-like giant cell tumors, serous cystadenocarcinomas, mucinous cystadenocarcinomas, intraductal papillary-mucinous carcinomas, acinar cell carcinomas, pancreatoblastomas, solid-pseudopapillary carcinomas, and miscellaneous carcinomas including other extremely rare forms of epithelial carcinomas not mentioned above (Klöppel et al., 1996).

Ductal adenocarcinomas commonly arise in the head of the pancreas and are characterized by infiltrating neoplastic epithelium and intense desmoplastic reaction (Hezel et al., 2006; Hidalgo, 2010). This dense, poorly vascularized tumor stroma forms a microenvironment that actively interacts with tumor cells, promoting tumorigenesis (Chu et al., 2007; Mahadevan et al., 2007; Olive et al., 2009). The pancreatic stellate cells or myofibroblasts are essential players in the formation and turnover of the tumor stroma along with fibroblasts, endothelial cells and inflammatory cells (Hildalgo, 2010). For example, pancreatic stellate cells secrete collagen and other matrix components in response to various growth factors (Masamune et al., 2009). The importance of the interplay between tumor cells and the surrounding microenvironment was not understood until recently (reviewed by Polyak et al., 2009). The tumor stroma serves not only as a mechanical barrier but also as a dynamic compartment that is involved in the whole process of tumor formation from initiation to metastasis (Chu et al., 2007; Mahadevan et al., 2007). The role of the tumor stroma in chemoresistance of PDAC was convincingly demonstrated by Olive et al. (2009) in a work where they reduced the tumor stroma in a mouse model by treatment with a hedgehog inhibitor, which led to improved delivery of gemcitabine and extended overall survival. Vascular, lymphatic, and perineural invasion along with infiltration of the spleen and peritoneal cavity are characteristic of pancreatic cancer (Hansel et al, 2003; Hezel et al., 2006). Pancreatic tumors typically metastasize to the liver and lungs (Hansel et al, 2003; Hezel et al., 2006).

PDAC is generally thought to originate from pancreatic ductal cells because of their morphological similarity, although the cell of origin is currently not known (Bardeesy & DePinho, 2002; Hezel et al., 2006). PDAC typically manifests as duct-like structures with varying degrees of cellular atypia (Baumgart et al., 2005; Hezel et al., 2006). Valuable information pertaining to disease pathogenesis has emerged from morphological examinations of proliferative precursor lesions and mouse

models of PDAC, supporting the hypothesis of a ductal origin (Mihaljevic et al., 2010). However, other pancreatic cell types, such as acinar cells, can also transdifferentiate into ductal-like tumor cells, providing an alternative route to PDAC (Parsa et al., 1985; Mihaljevic et al., 2010). This hypothesis has been further strengthened by studies in transgenic mice (Sandgren et al., 1990; Wagner et al., 1998). In addition, a small subgroup of pancreatic cancer cells (less than 5%) has been found to have cancer stem cell (CSC)-like properties that render them capable of asymmetric cell division, thus enabling them to generate both mature cells and cancer stem cells (Li et al., 2007; Hermann et al., 2007). The question arises whether these cells can give rise to pancreatic cancer. The idea of cancer stem cells arose about a decade ago as minor subpopulations of cells within tumors were observed to drive tumorigenesis and possess some characteristics of stem cells (Reya et al., 2001). Recently, pancreatic cancer stem cells were reported to express stem cell-specific surface markers, exert properties of self-renewal and multilineage differentiation, and show upregulation of developmental genes such as *Sonic hedgehog* (*SHH*) and *BMI-1* (Lee et al., 2008). The ongoing debate about cancer stem cells may also pertain to pancreatic cancer.

Pancreatic cancer has been suggested to follow a genetic progression model (Figure 3) according to which it evolves from benign epithelium to fully invasive cancer via premalignant lesions called pancreatic intraepithelial neoplasms (PanINs), in parallel with successive accumulation of genetic aberrations (Hruban et al., 2000). PanINs exert a spectrum of distinctive morphological changes compared to normal ductal epithelium that are suggested to represent gradual stages of dysplastic growth (Hruban et al., 2000). This progression model has recently been augmented with accompanying desmoplastic changes in the surrounding stroma that contribute to malignant transformation (Morris et al., 2010). PanINs are the most common and well-studied precursor lesions of the pancreas; other precursor lesions include mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) (Brugge et al., 2004; Maitra et al., 2005; Hezel et al., 2006). MCNs are large, mucin-producing cystic lesions with a distinctive ovarian-type stroma and variable degree of epithelial dysplasia (Brugge et al., 2004; Maitra et al., 2005; Maitra & Hruban, 2008), whereas IPMNs resemble PanINs morphologically but grow into larger cystic structures (Hezel et al., 2006; Maitra & Hruban, 2008).

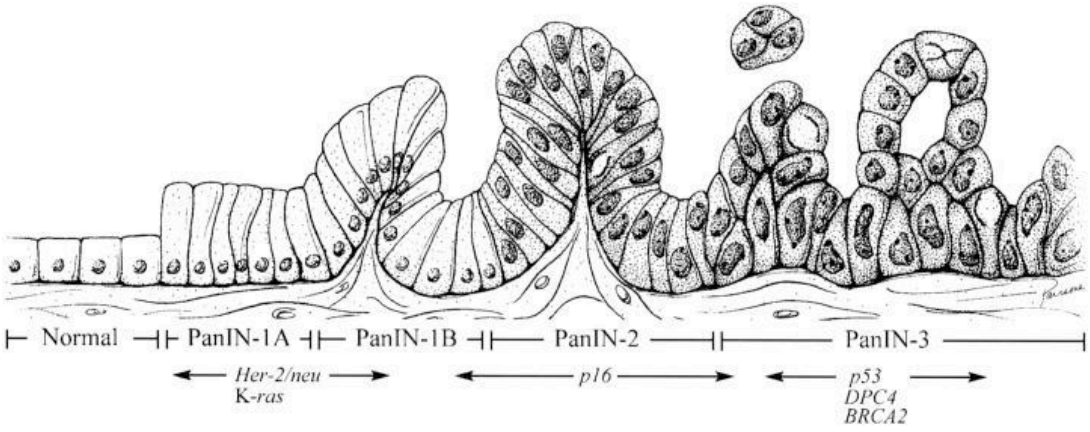

Figure 3. *Progression model for pancreatic cancer (Hruban et al., 2000). The tumor progression from normal epithelium towards carcinoma in situ occurs through precursor lesions called pancreatic intraepithelial neoplasias (PanINs), which are linked to specific genetic alterations. Adapted from Am J Pathol 2000, 156:1821-1825 with permission from the American Society for Investigative Pathology.*

2.3. Genetic alterations in pancreatic cancer

Pancreatic cancer is a disease of germline and somatic genetic alterations. Given that approximately 10% of pancreatic cancers are estimated to derive from inherited predisposition (Lynch et al., 1996), the vast majority of alterations are sporadic. Data suggest stepwise accumulation of specific genetic changes in ductal epithelium together with evolving morphological stages (Figure 3) (Hruban, 2000). Alterations targeting specific genes along with increasing cellular atypia include activation of *HER-2/neu* (also known as *ERBB2*) and *KRAS* oncogenes and inactivation of *CDKN2A/p16*, *TP53*, *BRCA2*, and *SMAD4/DPC4* tumor suppressor genes (Bardeesy & DePinho, 2002; Baumgart et al., 2005; Maitra & Hruban 2008; Hezel et al., 2006). The *HER-2* oncogene is amplified and overexpressed in a variety of solid tumors, including pancreatic cancer (Day et al., 1996; Apple et al., 1999; Mahlamäki et al. 2002), but this aberration is not the most typical alteration in PDAC. Instead, activating point mutations of the *KRAS* oncogene are among the most common genetic changes in pancreatic cancer, occurring in about 90% of cases (Maitra & Hruban, 2008; Hidalgo, 2010). *KRAS* mutations arise in an early low-grade PanIN lesion (PanIN-1) (Figure 3), leading to constitutively activated Ras protein and activation of several proliferative and survival signaling pathways. Similarly, 95% of pancreatic tumors have inactivating mutations in the *CDKN2A*

tumor suppressor gene, resulting in the loss of the growth regulatory protein p16 and the subsequent loss of cell cycle control (Maitra & Hruban, 2008). *CDKN2A* mutations occur in intermediate lesions (PanIN-2) and are followed by inactivating mutations in *SMAD4*, *TP53*, and *BRCA2* in late lesions (PanIN-3) (Figure 3). *SMAD4* or *DPC4* (deleted in pancreatic cancer) is inactivated in 50% of pancreatic tumors by homozygous deletion or intragenic mutation. SMAD4 is involved in TGFβ signaling and provides tumor cells with a selective growth advantage when abrogated (Siegel et al., 2003). *TP53* is inactivated in 50-75% of pancreatic cancers, leading to abnormal cell division and survival with further accumulation of genetic defects (Vogelstein & Kinzler, 2004). Loss of *BRCA2* affects approximately 10% of pancreatic carcinomas, giving rise to increased genomic instability, which is characteristic of PDAC. Telomere shortening is also a typical feature of pancreatic cancer, occurring at the early stages of progression (van Heek et al., 2002; Ranganathan et al., 2009). In addition, developmental pathways, including Hedgehog, Notch, and Wnt-β-catenin signaling pathways, are reported to become re-activated during PDAC tumorigenesis (Berman et al., 2003; Miyamoto et al., 2003; Zeng et al., 2006; Pasca di Magliano et al., 2007).

Several genetically engineered mouse models have been created to study pancreatic cancer progression, most of which are based on activated KRAS (reviewed by Morris et al., 2010). KRAS belongs to the RAS superfamily of GTPases and mediates a number of cellular functions from proliferation to differentiation and survival (Malumbres & Barbacid, 2003). It is nearly universally mutated in advanced PDACs and carries activating mutations at early premalignant stages. A conditional, genetically engineered, KRAS-driven mouse model has been shown to recapitulate the progression observed in humans from PanIN lesions to invasive PDAC (Hingorani et al., 2003). This process is further accelerated if the model is combined with the inactivation of appropriate tumor suppressors, such as *CDKN2A* (Aguirre et al., 2003), *TP53* (Hingorani 2005) and *SMAD4* (Bardeesy et al., 2006). The role of different developmental pathways in PDAC development has been actively studied using transgenic mice (reviewed by Morris et al., 2010). These KRAS-driven models have provided valuable knowledge about the initiation and progression of PDAC.

Genomic changes in pancreatic cancer include multiple types of aberrations from losses and gains of whole chromosomes to changes at selected chromosomal

loci (Baumgart; 2005). Alterations are often frequent and heterogenous. Cytogenetic analysis of primary pancreatic tumors and cell lines have revealed multiple, nonrandom numerical and structural chromosomal abnormalities (Johansson et al., 1992; Bardi et al., 1993; Griffin et al., 1995; Höglund et al., 1998) including translocations, chromosomal breaks, and amplifications. Highly complex karyotypes with various aberrations and large intratumoral heterogeneity are often observed (Griffin et al., 1995; Gorunova et al., 1998). Studies using conventional CGH as well as recent array-based CGH methods have revealed several chromosomal regions of recurrent losses and gains in pancreatic cancer including losses of 3p, 4q, 6q, 8p, 9p, 13q, 18q, and 22q and gains of 5p, 7p, 7q, 8q, 11q, 12p, 17q, 19q and 20q (Mahlamäki et al., 1997, 2004, Gorunova et al., 1998; Schleger et al., 2000; Harada et al., 2002; Aguirre et al., 2004; Heidenblad et al., 2004; Gysin et al., 2005; Nowak et al., 2005). Amplification of *AKT2* (a known oncogene in ovarian cancer) on chromosome 19q has been reported in 10%–15% of pancreatic cancers (Cheng et al., 1996; Miwa et al, 1996) and amplification of oncogenic *CMYC* on chromosome 8q has been reported in 20%–30% of cases (Nowak et al., 2005; Schleger, 2002). Other amplified and overexpressed putative oncogenes in pancreatic cancer, which play a significant role in tumor development based upon clinical or biological evidence (according to criteria presented by Santarius et al., 2010), include *ARPC1A* (Laurila et al., 2009) and *SMURF1* (Suzuki et al., 2008).

In addition to genetic and genomic aberrations in pancreatic cancer, gene expression patterns are also modulated by epigenetic events and miRNAs (as discussed earlier). Tumor suppressor genes are frequently inactivated by aberrant promoter hypermethylation in multiple cancers. Many of them, including *CDKN2A*, *MLH1*, and *CDKN1C*, have been reported to be silenced by hypermethylation in pancreatic cancer (Ueki et al., 2000; Omura & Goggins, 2009). Recent miRNA profiling by Lee and colleagues identified unique miRNA profiles in normal, benign and malignant pancreatic tissues (Lee et al., 2007). Another miRNA profiling in pancreatic cancer was able to differentiate normal pancreas, chronic pancreatitis and PDAC based on their miRNA profiles (Bloomston et al., 2007).

As already described, pancreatic cancer is known to be a multifaceted disease with mixed genetic background. This finding was recently confirmed by a comprehensive genetic analysis, which profiled somatic mutations of 20,661 protein-coding genes in 24 pancreatic ductal adenocarcinomas (Jones et al., 2008).

This detailed genetic profiling firmly indicated pancreatic cancer to be an extremely complex and heterogenous malignancy, harboring an average of 63 genetic abnormalities per tumor. However, this complexity was considerably reduced when the alterations were organized into processes and pathways. Despite the large number of identified changes and case-to-case variation, each of the aberrations seemed to fall into one of 12 common pathways (Figure 4). This pattern simplifies the complexity of pancreatic cancer and guides us towards a pathway-oriented strategy instead of focusing on individual genes and proteins.

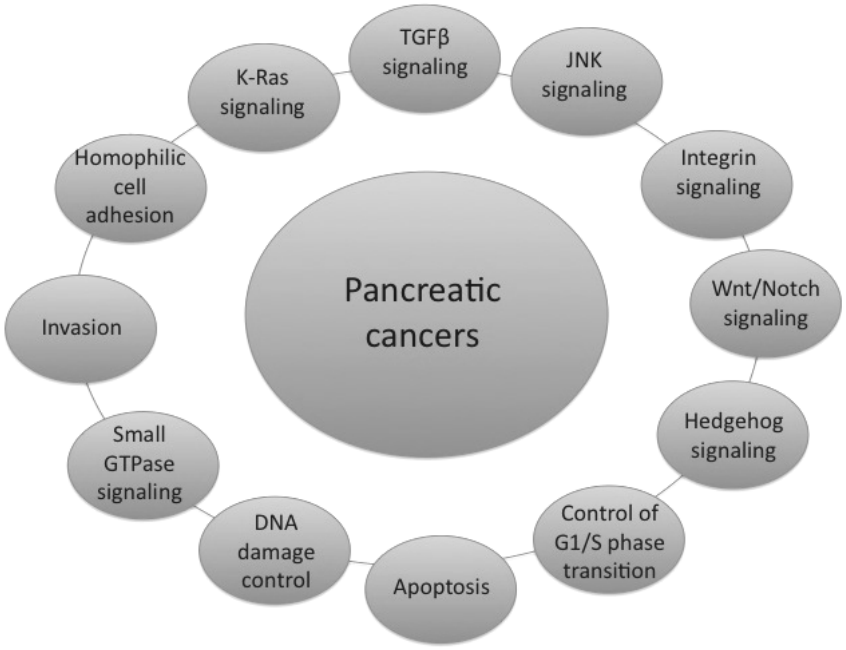

Figure 4. *Signaling pathways and processes involved in pancreatic cancer (modified with permission from Jones et al., 2008). The genetically altered genes in pancreatic carcinomas typically fall into these twelve pathways and processes presented here.*

2.4. Clinical presentation, diagnosis and treatment

Pancreatic cancer remains a substantial therapeutic challenge due to the lack of a means for early detection and resistance to current treatment strategies. Symptoms arise late and less than 20% of patients are diagnosed with a localized, potentially curable tumor (Freitas et al., 2009; Hidalgo, 2010). Symptoms are often broad and

depend on the location of the tumor and the stage of the disease. Vague abdominal pain and nausea are common non-specific symptoms. Tumors that develop in the head of the pancreas can cause obstructive cholestasis, leading to painless jaundice. Systemic manifestations of the disease include asthenia, anorexia, and weight loss (Freitas et al., 2009; Hidalgo, 2010). Physical examination may provide additional information, but results from routine blood tests are generally non-specific. Mild abnormalities in liver function tests, hyperglycemia and anemia may be found (Hidalgo, 2010).

The initial evaluation of pancreatic cancer is often done using multidetector computed tomography (CT) or magnetic resonance imaging (MRI) with or without fine needle biopsy to visualize the primary tumor and to outline an initial management plan (Freitas et al., 2009; Hidalgo, 2010). Endoscopic ultrasonography, or, more rarely, endoscopic retrograde cholangiopancreatography (ERCP) may be used if the tumor is not identifiable on CT or if a tissue biopsy is needed (Hidalgo, 2010). ERCP is associated with an increased risk of complications and is now rarely used without a therapeutic intent. There are many potential serum biomarkers to ease diagnosis, but only carbohydrate antigen CA 19-9 has demonstrated clinical usefulness (Harsha et al., 2009). Nevertheless, even CA 19-9 has its limitations, such as a lack of precision and, more rarely, false negative results.

Operative procedures include cephalic pancreatoduodenectomy, distal pancreatectomy, and total pancreatectomy. Gemcitabine alone or in combination is considered the standard post-operative treatment (Freitas et al., 2009; Hidalgo, 2010). According to recent data, one out of eight patients who undergoes surgical resection lives for longer than ten years (Schnelldorfer et al., 2008). In addition, the three-year survival rate for patients with resectable PDAC in Finland during the years 2000-2006 was 29% (Kiviluoto, 2008). Treatment of patients with locally advanced or metastatic disease is mainly palliative. Possible treatment strategies include chemotherapy or combined chemoradiation therapy (Hidalgo, 2010). In addition, there is on-going clinical development of new targeted agents, including small molecule inhibitors, monoclonal antibodies, nanotechnology, and adenoviral agents. We should nevertheless keep in mind the importance of early diagnosis and detection of neoplastic precursors as the most effective means to reduce cancer-related mortality, as has been shown with other malignancies (Andriole et al., 2005; Saslow et al., 2007; Levin et al., 2008).

3. Transcriptional regulation

3.1 Mechanisms of transcription

The regulation of transcription is a key step in maintaining cellular homeostasis and controlling cellular growth, differentiation, and development (Hahn, 2004; Koch et al., 2008). This level of regulation is mediated by a number of transcription factors and coregulators that activate or repress transcription in response to numerous signals originating from inside or outside the cell (Conaway et al., 2005b). Three main RNA polymerases (pol) are responsible for nuclear transcription in eukaryotes: RNA polymerases I and III transcribe noncoding genes, such as those encoding transfer RNAs or ribosomal RNAs, whereas RNA polymerase II transcribes coding mRNAs and some noncoding RNA species (Koch et al., 2008).

Eukaryotic mRNA synthesis is a stepwise process driven by RNA polymerase II and regulated by specific activator and repressor proteins, general transcription factors, and a coregulatory multiprotein complex, Mediator, which mediates regulatory signals from specific regulators to pol II and the general transcription factors at the core promoter (Conaway et al., 2005b; Kornberg, 2005). The core promoter is the DNA sequence where pol II and the general transcription factors assemble. The general transcription factors involved in pol II-mediated transcription include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. They help to position the polymerase at the promoter and facilitate transcription (Figure 5). The assembly starts with TFIID recognizing a specific binding site in the DNA, such as the TATA-box, a TA-rich region at the promoter, usually located 25 base pairs upstream of the transcription start site. TFIIA stabilizes TBP (a TATA-box binding protein, a part of the TFIID complex) and TFIID binding to DNA (Weideman et al., 1997) and blocks some transcription inhibitors from binding to DNA (Kokubo et al., 1998; Hahn, 2004). TFIIB binds to DNA-bound TBP and later to pol II, facilitating formation of the preinitiation complex (PIC) (Hahn & Roberts, 2000), which is an inactive state of Pol II and associated factors assembled at the core promoter just before transcription initiation. TFIIF binds to Pol II, facilitating its recruitment to the promoter and PIC formation. TFIIE and TFIIH function primarily during the steps after PIC formation. TFIIH melts the DNA, thus allowing pol II access to the

DNA template. TFIIH has both kinase and helicase activities, and TFIIE is thought to support TFIIH in its function (Ohkuma, 1997).

Pol II recruitment to DNA is the most important step in transcriptional regulation that can be facilitated or repressed by specific activators or repressors, respectively. Specific activator or repressor proteins typically bind distant from the transcriptional start site and mediate their signals via coregulatory complexes such as the Mediator complex (Koch et al., 2008; Ansari et al., 2009). The regulatory sequences or elements (RE), for example, upstream activating sequences (UAS), serve as binding sites for specific regulatory proteins and can locate adjacent to the promoter but also further upstream or downstream of it (Taatjes et al., 2004; Malik & Roeder, 2005). The accessibility of these binding locations is regulated by a wide spectrum of post-translational histone modifications, making the regulation even more complex (Taatjes et al., 2004). Histone acetyltransferases (HAT) are enzymes that acetylate lysine residues on histones, diminishing the interaction between histones and the DNA and rendering the DNA more accessible to transcription factors. The composition of the involved proteins and protein complexes and their interactions during PIC formation are illustrated in Figure 5.

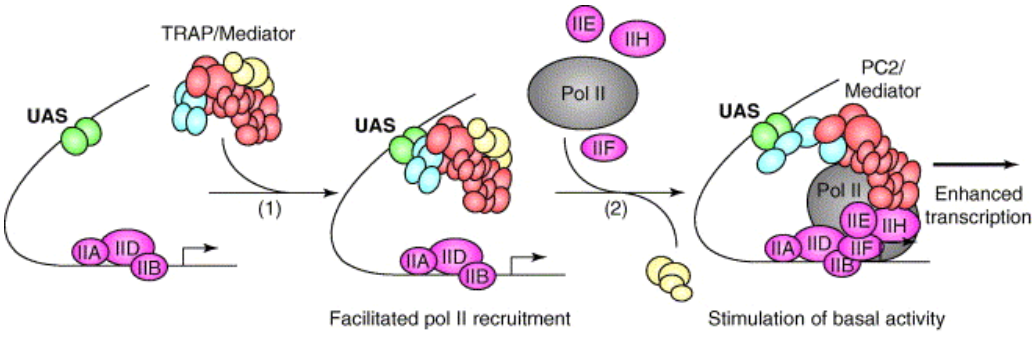

Figure 5. *Model for PIC formation and activated transcription. Transcriptional activators bind to their target sites (upstream activated sequence, UAS), recruiting Mediator, which in turn facilitates the pol II recruitment to the core promoter and the PIC formation, leading finally to initiation of transcription. Mediator acts here as a bridge between activators and the general pol II transcription machinery including pol II and the general transcription factors (IIA-IIH). (Reprinted with permission from Malik & Roeder, 2005).*

3.2 Mediator in transcription

Mediator plays a fundamental role in the activation and repression of eukaryotic mRNA synthesis and is presumed to be an important component of the RNA polymerase II general transcription machinery (Kim et al., 1994; Thompson et al., 1995; Takagi et al., 2006; Conaway et al., 2005a). It bridges the signals between specific transcriptional regulators and the general transcription apparatus (Taatjes et al., 2004), but the actual mechanism of action of mammalian Mediator has not been firmly established (Conaway et al., 2005a).

Mediator was originally identified in *Saccharomyces cerevisiae* through the discovery that purified activators and the general transcription machinery alone were incapable of active transcription *in vitro* (Flanagan et al., 1991). Later, the yeast Mediator was purified to homogeneity and found to stimulate transcription in the absence of an activator (Kim et al., 1994). Further functional studies on Mediator have reported both stimulatory and inhibitory functions *in vitro* (Conaway et al., 2005a). *In vivo*, Mediator seems to be a direct target of activators and is recruited to gene promoters at the preinitiation stage of transcription to facilitate pol II recruitment and PIC formation (Figure 3) (Bhaumik et al., 2004; Bryant & Ptashne, 2003; Govind et al., 2005; Kuras et al., 2003).

Mediator is structurally and functionally highly conserved across eukaryotes, comprising 25-30 subunits (Myers et al., 2000; Boube et al., 2002; Sato et al., 2004) that are organized into three distinct modules: head, middle and tail (Dotson et al., 2000; Chadick et al., 2005). Although the mechanism by which Mediator interacts with pol II is not fully understood, the yeast Mediator appears to bind to the C-terminal domain (CTD) of the polymerase (Kim et al., 1994; Thompson et al., 1993). Furthermore, it is now well documented that yeast Mediator interacts directly with transcriptional activation domains of several DNA binding regulatory proteins (Myers et al., 2000; Kim et al., 1994; Malik & Roeder, 2000). Of note, different subunits of Mediator appear to interact with different transcriptional activators (Conaway et al., 2005b), and Mediator complexes with distinct kinase modules may regulate different sets of genes (Sato et al., 2004). These findings suggest a complex and multidimensional role for Mediator in transcriptional regulation.

3.3 The MED29 subunit

Human *MED29* (previously known as Intersex-like, *IXL*) encodes a protein of 221 amino acids, which is a subunit located in the head module of the Mediator complex (Sato et al., 2003b). Mammalian MED29 was identified as a novel, previously uncharacterized subunit of the Mediator complex by tandem mass spectrometry analysis of highly enriched Mediator fractions of rat liver nuclei (Sato et al., 2003b). The same subunit was identified and characterized from a human embryonic heart cDNA library and called Intersex-like (IXL) due to its close homology to Drosophila melanogaster intersex protein (Wang et al., 2004). After these initial discoveries of mammalian MED29, no further studies on its functional role have been published. The role and mechanism of action of MED29 in transcriptional regulation remain elusive.

MED29 is widely expressed during different stages of embryonic development and in various human adult tissues (Wang et al., 2004). Sequence analysis shows high evolutionary conservation across species, with over 90% identity with mouse Ixl and 36% identity with Drosophila melanogaster Intersex (Wang et al., 2004). Drosophila melanogaster intersex is an important transcriptional coregulator that interacts with a DNA-binding transcriptional activator doublesex and is involved in female somatic sex determination (Garrett-Engele, 2002). Similarly, MED29 may be a coregulatory target of some DNA-binding transcriptional regulators. MED29 contains a consensus Src homology 3 (SH3) binding site for the Nck adaptor protein, which suggests a role in downstream signaling cascades, such as Pak and mitogen-activated protein kinase (MAPK) pathways (Wang et al., 2004). MAPK pathways are main signaling cascades through which cells transduce signals outside of the cell into intracellular responses involving growth, differentiation, damage repair, and cell death.

In COS-7 cells, MED29 is predominantly localized in the nucleus, but it is also found in the cytoplasm (Wang et al., 2004). The role of MED29 in transcriptional regulation fits well with its nuclear localization. MED29 was reported to act as a transcriptional suppressor when fused with a Gal4-DNA-binding domain and cotransfected with VP-16 into COS-7 cells (Wang et al., 2004). Overexpression of MED29 was shown to significantly inhibit the transcriptional activities of *SRE* and *AP-1*, the main downstream targets of MAPK signaling, suggesting a

downregulatory role of MED29 in the MAPK signaling cascade in COS-7 cells (Wang et al., 2004). MAPK signaling affects four different subtypes of MAPKs: extracellular signal-related kinases (ERK1/2), Jun amino-terminal kinases (JNK1-3), p38 proteins, and ERK5 proteins, being thus in an essential and broad role in the control of cellular growth and differentiation (Chang et al., 2001). These initial discoveries suggest that MED29 could play a role in the regulation of cell growth and development, but further studies are needed to confirm these findings and to define the mechanisms of action.

AIMS OF THE STUDY

The aims of this study are to characterize the recurrently amplified chromosomal region at 19q13 in pancreatic cancer, to identify putative novel targets in this region and to evaluate their clinical significance. The specific aims are as follows.

1. To define the core region of the 19q13 amplification in pancreatic cancer and to identify putative novel target genes within the core amplicon.
2. To evaluate the clinical significance of the 19q13 core amplicon in large patient material.
3. To characterize the functional role of the putative amplification target genes using gene silencing and gene transfer techniques.

MATERIALS & METHODS

1. Cell lines (I, III)

A total of 16 pancreatic cancer cell lines were included in the study. AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAC, HPAF-II, Hs 700T, Hs 766T, MIA PaCa-2, PANC-1, SU.86.86, and SW1990 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DAN-G, HUP-T3, and HUP-T4, were obtained from the German Collection of Microorganisms and Cell Cultures (Brunswick, Germany). The breast cancer cell lines BT-474 and MCF7, the human embryonic kidney cells (HEK 293T/17), and the mouse embryonic fibroblast cell line (NIH/3T3) were obtained from the ATCC. Bacterial TOP10 *Escherichia coli* cells were obtained from Invitrogen (Carlsbad, CA). All cell lines were grown under recommended conditions.

2. Tumor samples (II)

The primary pancreatic tumor samples and the corresponding metastases were obtained from 356 pancreatic surgery patients at the Department of General-Visceral- and Thoracic Surgery, the University Medical Center Hamburg-Eppendorf during the years 1993-2005. The samples were fixed with formalin and embedded in paraffin. All tumors were reviewed by two pathologists who determined the histological type and grade of the samples (G1 = highly differentiated, G2 = moderately differentiated, G3 = poorly differentiated). The pathologic stage, nodal status and metastasis information were included as well as the follow-up and survival data. The standard TNM classification for pancreatic cancer was used: pT stage T1 = tumor size $\leq$ 2 cm, T2 = tumor size > 2 cm, T3 = tumor growth into surrounding tissues, T4 = tumor growth into the stomach, spleen, large bowel or nearby large blood vessels; pN stage N0 = no lymph node metastases, N1 =

metastases in local lymph nodes; and pM stage M0 = no distant metastases, M1 = distant metastases. The median age of the patients was 62.8 years (range 21-88 years) and the mean follow-up time for ductal adenocarcinomas was 18.52 months (range 1-74 month). Informed consent had been obtained from all patients upon admission to hospital.

3. Tissue microarrays (I, II)

Two different pancreatic cancer tissue microarrays (TMAs) and one cell line microarray were used for the DNA copy number analysis in this study. The first TMA was a commercial array obtained from Petagen Incorporation (Seoul, Korea) and contained 33 pancreatic cancer samples and four non-neoplastic pancreatic tissue specimens. The other TMA was build from the patient material described above and contained a total of 600 samples, including 357 primary tumors of the pancreas (213 ductal adenocarcinomas, 54 adenocarcinomas of the ampulla of Vater, 40 pancreatic endocrine tumors, 33 intraductal papillary mucinous neoplasms (IPMNs), 15 benign cystic tumors, one malignant cystic tumor, and one acinar cell carcinoma), 129 corresponding lymph node metastases, 22 distant metastases, 24 local recurrences, and a standard control area containing 40 tumors from other organs, 10 healthy pancreatic tissue samples, and 18 healthy tissue samples of other sites. The cell line microarray contained in total 120 cancer cell lines representing various tissue types and nine cell lines of non-neoplastic origin. The content of the smaller pancreatic tissue array used in the Study I is described in Table 2.

Table 2. *Clinicopathological data of the tumor specimens on the TMA in the Study I.*

No	Sex	Age	Histology	Grade	TNM Stage
1	M	66	Non-neoplastic region		
2	M	71	Non-neoplastic region		
3	M	85	Non-neoplastic region		
4	M	71	Non-neoplastic		
5	M	49	Ductal adenocarcinoma	G1	T4N3M0 (III)
6	M	61	Ductal adenocarcinoma	G3	T3N1M1 (IV)
7	M	65	Ductal adenocarcinoma	G3	T2N4M1 (IV)
8	M	66	Ductal adenocarcinoma	G1-G2	T3N1M0 (IIB)
9	M	55	Ductal adenocarcinoma	G1-G2	T2N1M0 (IIB)
10	M	71	Ductal adenocarcinoma	G1-G2	T1N1M0 (IIB)
11	M	59	Ductal adenocarcinoma	G3	T3N0M0 (IIA)
12	M	60	Ductal adenocarcinoma	G3	T3NXM0 (IIA)
13	M	71	Ductal adenocarcinoma	G2	T3N1M0 (IIB)
14	M	85	Ductal adenocarcinoma	G2	T3N0M0 (IIA)
15	M	55	Ductal adenocarcinoma	G2	T3N0M0 (IIA)
16	F	69	Ductal adenocarcinoma	G3	T4N1M1 (IV)
17	M	66	Ductal adenocarcinoma	G3	T3N0MX (IIA)
18	F	78	Ductal adenocarcinoma	G2	T3N1M0 (IIB)
19	M	47	Ductal adenocarcinoma	G2	T3N1M1 (IV)
20	M	65	Ductal adenocarcinoma	G2	T2N0M1 (IV)
21	M	61	Ductal adenocarcinoma	_	T2N1M1 (IV)
22	M	69	Ductal adenocarcinoma	_	T3N0M0 (IIA)
23	F	78	Ductal adenocarcinoma	G2	T2N0M0 (IB)
24	M	66	Ductal adenocarcinoma	G2	T3N1M1 (IV)
25	M	64	Ductal adenocarcinoma	G2	T3N1M0 (IIB)
26	M	69	Ductal adenocarcinoma	_	T3N0M1 (IV)
27	F	66	Ductal adenocarcinoma	_	T3N0M0 (IIA)
28	M	64	Ductal adenocarcinoma	G2	T3NXM1 (IV)
29	M	60	Ductal adenocarcinoma	G2	T3N1M0 (IIB)
30	M	63	Ductal adenocarcinoma	G2	T3N1M0(IIB)
31	M	74	Ductal adenocarcinoma	_	T3N0M0 (IIA)
32	M	43	Endocrine carcinoma	_	
33	M	60	Ductal adenocarcinoma	G2	T3N0M0 (IIA)
34	M	75	Ductal adenocarcinoma	G2-G3	T2N1MX (IIB)
35	F	46	Ductal adenocarcinoma	G2-G3	T3N1M1 (IV)
36	F	65	Endocrine carcinoma		
37	F	60	Ductal adenocarcinoma	G4	T3N1M1 (IV)

4. Genomic clones (I, II)

Fifteen evenly distributed bacterial artificial chromosome (BAC) clones over the entire 2.9 Mb amplicon at 19q13 were selected for the copy number analysis using public genome databases (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov and University of California Santa Cruz Genome Bioinformatics: http://www.genome.ucsc.edu). The BAC clones were purchased from Invitrogen (Carlsbad, CA), whereas the pericentromeric reference clone RP11-

345J21 was a kind gift from Mariano Rocchi (the University of Bari, Bari, Italy). The list of all BAC clones is shown in Table 3.

Table 3. *Genomic BAC clones used in the copy number analysis by FISH. Localization is based on UCSC Genome Browser assembly May 2004.*

Clone name	GenBank accession	Size (kb)	Start (bp)	End (bp)
CTB-186G2	AC008649	127	43772845	43893312
CTD-2540F13	AC022144	149	43893313	43987758
CTC-360G5	AC011455	149	44037170	44178197
CTC-218B8	AC011443	157	44244816	44401384
RP11-67A5	AC018477	172	44461580	44633655
RP11-256O9	AC016381	76	44600000	44700000
CTC-488F21	AC139454	191	44934122	45124860
RP11-746O9	AQ450800, AQ453825 [a]	183	45113627	45297066
RP11-246P10	AQ488066, AQ488067 [a]	170	45304261	45473822
CTC-425O23	AC118344	178	45390096	45568430
CTC-492K19	AC010271	161	45566202	45663017
CTD-2223D2	AC020929	120	45661273	45780841
CTC-490E21	AC008537	169	45975835	46143755
CTD-2356P16	AC008962	154	46227483	46373579
CTD-2195B23	AC011510	129	46373580	46498596
RP11-345J21 [b]	AQ540013, AQ540014 [a]	196	34827676	35023872

[a] BAC end sequences, [b] pericentromeric control clone

5. Fluorescence in situ hybridization (I, II)

Fluorescence in situ hybridization (FISH) was used for copy the number analysis of the 19q13 locus in pancreatic cancer cell lines and three different TMAs described above. All the 19q13 BAC clone DNA probes were labeled with SpectrumOrange-dUTP (Vysis, Downers Grove, IL) and the pericentromeric reference probe (RP11-345J21) was labeled with fluorescein-12-dUTP (Perkin-Elmer, Boston, MA) by random priming. Dual-color FISH was performed as previously described (Bärlund et al., 2000) and hybridization signals were evaluated using the Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan). Forty intact nuclei were scored for each probe, and relative copy numbers were calculated as the ratios of mean absolute copy number of the test probe versus the reference probe.

For the cell lines, each BAC clone was individually hybridized against the reference probe, whereas a contig of three overlapping BAC clones (RP11-67A5, RP11-256O9, and CTC-488F21), located in the core region of the 19q13 amplicon, was used for the TMAs and the cell line array. Control experiment on normal

lymphocytes verified that this probe combination gave a single hybridization signal. The RP11-345J21 probe was again used as a reference.

FISH on tissue microarrays was carried out as described (Andersen et al., 2001) with slight modifications. Briefly, the slides were deparaffinized in three changes of hexane for 10 min each, dipped twice in 100% EtOH, treated with 0.3% NaBH4 for 30 min, and rinsed with PBS. Then the slides were incubated in the Vysis Pretreatment Solution (Vysis, Downers Grove, IL) for 40 min at 80°C, rinsed with H_2O, and treated with the Vysis Protease for 20 min at 37°C. This was followed by the Proteinase K treatment for 10 min at 37°C. Finally the slides were washed in the increasing series of EtOH (70%, 85%, 100%), dehydrated, denatured in 70% formamide/2X SSC for 3 min at 70°C, washed with EtOH, dehydrated, and hybridized with the denatured probes in a humidified chamber at 37°C for 24h. The nuclei were counterstained with DAPI in Vectashield antifade solution.

The hybridization signals were scored and evaluated as described above. Relative copy numbers greater than or equal to 2 were considered as amplifications whereas copy numbers over 1.5 but less than 2 were considered as gains. Polysomy was defined as samples with the absolute copy number of the 19q13 locus over 5 but the relative copy number less than two.

6. Quantitative RT-PCR (I, III)

Gene expression analyses for all 23 known genes in the 1.1 Mb core amplicon that were expressed in PANC-1 cells were performed using Light Cycler equipment (Roche, Mannheim, Germany). Further gene expression validations were performed with the Light Cycler or the ABI 7900HT Fast Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA was isolated from cell lines using the RNeasy Mini kit (Qiagen) and transcribed into first-strand cDNA using Superscript III reverse transcriptase (Invitrogen) or the Script cDNA synthesis kit (Bio-Rad, Inc., Hercules, CA). Xenograft RNA was extracted by mechanical lysis using the MagNA Lyser instrument (Roche Diagnostics GmbH, Mannheim, Germany) and was further purified by use of the RNeasy Mini kit (Qiagen), similarly to RNA harvested from cell lines. Normal human pancreatic RNA was obtained from Ambion (Cambridgeshire, United Kingdom). For analyses using the Light Cycler

system, gene expression levels were normalized against house-keeping gene *TBP* (TATA-box binding protein) or *GUSB* (glucuronidase beta), and Light Cycler software (Roche) was used for data analysis. Primers and probe sets for the Light Cycler were obtained from TIB MolBio (Berlin, Germany), and the Universal ProbeLibrary assay for *GUSB* was obtained from Roche. TaqMan gene expression assays for the ABI system were obtained from Applied Biosystems, and ABI 7900HT software (Applied Biosystems) was used for data analyses. Expression levels of the target genes in the ABI system were normalized against the housekeeping gene *GAPDH* (glyceraldehyde- 3-phosphate dehydrogenase).

7. Sequencing (III)

CDNA from PANC-1 and SU.86.86 cells was PCR amplified using primers from both ends of the *MED29* coding sequence. The reaction contained 1 µl of cDNA, 5 µl of 10x PCR Gold Buffer, 4 µl of 25µM $MgCl_2$, 1 µl of dNTPs, 1 µl of each 10 µM primer, and 0.3 µl of Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The cycling program included 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 1 minute, and extension at 72º for 1 minute. The PCR products were purified using the Qiagen QIAquick PCR Purification Kit according to the manufacturer's instructions (Qiagen Inc.). The sequencing reactions were carried out using the Big Dye Terminator chemistry (Applied Biosystems) according to the manufacturer's protocol and the ABI 3100 sequencer (Applied Biosystems).

8. Western blot (III)

To collect total protein, cells were lysed into RIPA buffer (1% PBS, 1% nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with a complete mini protease inhibitor cocktail (Roche Diagnostics GmbH). The protein content was quantified using Bradford reagent (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Proteins were separated by SDS-PAGE and transferred onto PVDF membrane (Roche Diagnostics GmbH) with a Trans-blot® SD transfer apparatus

(Bio-Rad Laboratories, Hercules, CA). After blotting, the membrane was blocked overnight. Immunodetection was performed using a custom-made MED29-specific primary antibody (GenWayBiotech, San Diego, CA), and proteins were visualized by using a BM chemiluminescence western blotting kit (Roche Diagnostics GmbH). β-Tubulin 1 (Sigma-Aldrich Chemie GmbH) was used as a loading control.

9. Loss-of-function RNA inhibition screen (I)

Loss-of-function screening using high-throughput RNA inhibition (RNAi) technology was performed for all known genes in the 1.1 Mb core amplicon that were expressed in 19q13 amplified PANC-1 cells. A small interfering RNA (siRNA) library that targeted all known genes in the studied amplicon one by one, was utilized (Table 3). Two genes, *SAMD4B* and *EID2B*, were hypothetical proteins at the time of the experiment and were thus not included. Four siRNAs were designed for each gene according to previously described criteria (Meister et al., 2004; Reynolds et al., 2004). All gene-specific siRNAs for the loss-of-function screen as well as a non-silencing control siRNA were obtained from Qiagen. Reverse siRNA transfection using Oligofectamine (Invitrogen) was applied on a 384-well Costar microtiter plate (Fisher Scientific, Hampton, NH). After complex formation, suspensions of MIA PaCa-2 or PANC-1 cells were added. The total cell number was quantified using the Cell Titer Blue method (Promega, Madison WI) and an EnVision plate reader (Perkin- Elmer, Wellesley, MA). Reduced cell viability to 50% or less compared with non-silencing control siRNA-treated cells was regarded as a significant change.

10. siRNA-mediated gene silencing (I, III)

Gene silencing by gene-specific siRNAs was used to study the functional role of *MED29* in pancreatic cancer cell lines with MED29 amplification and subsequent overexpression. Silencing was obtained with the single siRNAs described above (Table 4) or by an ON-TARGETplus SMART siRNA pool containing a mixture of four gene-specific siRNAs specific for MED29 (Dharmacon, Lafayette, CO). An siRNA targeting the firefly luciferase gene (LUC) (Sigma, St. Louis, MO) was used as a control. Interferin transfection reagent was used for siRNA transfection according to the manufacturer's instructions (Polyplus Transfection, San Marcos, CA). Transfections were performed in standard growth medium. The efficiency of MED29 silencing in each experiment was confirmed by quantitative RT-PCR.

Table 4. *The siRNA sequences used in the study.*

Gene	Gene Bank	siRNA	Antisense	Sense
GMFG	XM_290842	198	UGUAAACCACGAACCUGGGct	CCCAGGUUCGUGGUUUAC
		243	ACAAAGGGUAGGACACUCGg	CGAGUGUCCUACCCUUUG
		27	UUAGCUCUGGGUCUACCUCg	GAGGUAGACCCAGAGCUA
		207	ACUUGUAGCUGUAAACCACg	GUGGUUUACAGCUACAAG
LRFN1	NM_004877	2069	AUAAUUACACGAAUCUCCCat	GGGAGAUUCGUGUAAUUA
		188	AUACAAGUGAAGGUGCCACt	GUGGCACCUUCACUUGUA
		754	UAUAGCGGAUCAUGAGCAGa	CUGCUCAUGAUCCGCUAU
		762	AUACACCUUAUAGCGGAUCat	GAUCCGCUAUAAGGUGUA
PD2	NM_019088	555	UCUUUCGCAUCCAUGGCACc	GUGCCAUGGAUGCGAAAG
		1051	ACGUUCCAGUUGUACUCCCg	GGGAGUACAACUGGAACG
		267	UAUUGCAGUACUUGACUCGg	CGAGUCAAGUACUGCAAU
		587	AUAACGGUUGAACUCAGUGg	CACUGAGUUCAACCGUUA
MED29	NM_017592	261	UAUAACGCUGCACAGGAUCg	GAUCCUGUGCAGCGUUAU
		584	AAUCUGGGCUUUGAUGACCg	GGUCAUCAAAGCCCAGAU
		144	AACUAGGACCCGAUACACCa	GGUGUAUCGGGUCCUAGU
		264	UCUUAUAACGCUGCACAGGat	CCUGUGCAGCGUUAUAAG
ZFP36	NM_003407	1524	UCGUCAUAAAUAAAGGGCCc	GGCCCUUUAUUUAUGACG
		376	AGAAGGUCCGACAUAGCUCa	GAGCUAUGUCGGACCUUC
		890	UAUUCAUCAGGGUCGGAUCc	GAUCCGACCCUGAUGAAU
		488	AACUUGUGACAGAGUUCCGt	CGGAACUCUGUCACAAGU
PLEKHG2	NM_022835	3463	AUAUUGGUUGGGCUCUCGCt	GCGAGAGCCCAACCAAUA
		3219	AAGUUGUAAGAGCCGGCACc	GUGCCGGCUCUUACAACU
		1774	ACAUAAGGGUCCUUCACCGg	CGGUGAAGGACCCUUAUG
		1677	UUCUAGCAUCUCGAGGUCGa	CGACCUCGAGAUGCUAGA
RPS16	NM_001020	360	AUAUUUCUGGUAAUAGGCCa	GGCCUAUUACCAGAAAUA
		161	UUGAUGAGACCAUUGCCGCg	GCGGCAAUGGUCUCAUCA
		3008	UAAAUCUGGGCCACGUGACc	GUCACGUGGCCCAGAUUU
		313	UAGCAUAAAUCUGGGCCACg	GUGGCCCAGAUUUAUGCU
SUPT5H	NM_003169	2304	AUGAGUUCGUUGUCCCUCCg	GGAGGGACAACGAACUCA
		525	ACAUUAUCGAUAUUGGAGGc	CCUCCAAUAUCGAUAAUG
		2694	UAUUCUUCCUCAGCCCGUGa	CACGGGCUGAGGAAGAAU
		3363	UCAAUGCUCAGUAGGACGCc	GCGUCCUACUGAGCAUUG
TIMM50	NM_00100156	964	AACAAGGUCUCGAUGCCUGg	CAGGCAUCGAGACCUUGU
		1099	AUGUAUCUUGUGGCGUCCCg	GGGACGCCACAAGAUACA
		1123	AUAUAGACGACGCUCACAGtc	CUGUGAGCGUCGUCUAUA
		117	AUAUCCUUUACAUGGUGUCc	GACACCAUGUAAAGGAUA
DLL3	NM_016941	117	AAGAGUGGAUCUGCAGCUCg	GAGCUGCAGAUCCACUCU
		1968	AGAAGAUGGCAGGUAGCUCa	GAGCUACCUGCCAUCUUC
		1862	UUUCACGGACAGAAUCGAGg	CUCGAUUCUGUCCGUGAA
		2302	UAUAGGUGUUUCUAGAGAGa	CUCUCUAGAAACACCUAU
CRI2	NM_153232	685	AUUCGGCAUCAAACGCUGCtt	GCAGCGUUUGAUGCCGAA
		780	AAGUUCUUCUAUCAGAGGGtt	CCCUCUGAUAGAAGAACU
		621	AAGCUCUUGUAUUCUGCCGg	CGGCAGAAUACAAGAGCU
		796	UAAACUUAUCGCAACCAAGtt	CUUGGUUGCGAUAAGUUU
DYRK1B	NM_004714	599	AAUUUCGUAGCGCUCCAGCc	GCUGGAGCGCUACGAAAU
		1607	AUAUCGGUUGCUGUAGCGGt	CCGCUACAGCAACCGAUA
		778	AUAUAGUACUUCAUCUCCGt	CGGAGAUGAAGUACUAUA
		1345	UCUUUCGUCCUUCGUAGGGtc	CCCUACGAAGGACGAAAG
FBL	NM_001436	644	UUAAUGAGGUCACGGCCAGa	CUGGCCGUGACCUCAUUA
		948	UUCAUAUGGCUCAAGGGUCa	GACCCUUGAGCCAUAUGA
		807	AUUACGCAGGAAGGUGUGGg	CCACACCUUCCUGCGUAA
		819	AAAGUGUCCUCCAUUACGCa	GCGUAAUGGAGGACACUU
FCGBP	NM_003890	6395	AUUUGCAGGGUGACACUGCg	GCAGUGUCACCCUGCAAA
		5974	UUAACACGAAACCCGCGUCg	GACGCGGGUUUCGUGUUA
		12000	AAUGAGGACUGUCUGCUCCg	GGAGCAGACAGUCCUCAU
		780	AUCAUAGCGAGAUUGGGAGg	CUCCCAAUCUCGCUAUGA
PSMC4	NM_006503	200	AAAUUGUCCGAUGACCAGCg	GCUGGUCAUCGGACAAUU
		260	AUAAUAGUUGGAGCCUGUGg	CACAGGCUCCAACUAUUA
		596	UGUUGUGUGAUGUGCCACCg	GGUGGCACAUCACACAAC
		752	AUCGAAUCUCUUGGUGGCGa	CGCCACCAAGAGAUUCGA
ZNF546	NM_178544	192	AAAGUCAUUUGGAGGCCCGt	CGGGCCUCCAAAUGACUU
		2656	UUAUGCAUAGGACUUCCUCa	GAGGAAGUCCUAUGCAUA
		743	AUUAGCAUUUGACUAACGCa	GCGUUAGUCAAAUGCUAA
		2279	AAUGUAAGUCGAUAACUGCa	GCAGUUAUCGACUUACAU
MAP3K10	NM_002446	1719	AUCUUAUGCUCAAAGCCAGa	CUGGCUUUGAGCAUAAGA
		1351	UUCGAUGACUUCAAGCCGCtt	GCGGCUUGAAGUCAUCGA
		1326	AUGCUACCGAAAUCUGGCCg	GGCCAGAUUUCGGUAGCA
		2401	AAAGAAGAGGCCGUCGAGCc	GCUCGACGGCCUCUUCUU
AKT2	NM_001626	631	AUCUUAUGCUCAAAGCCAGa	CUGGCUUUGAGCAUAAGA
		1511	AAUUCAUCAUCGAAGUACCtt	GGUACUUCGAUGAUGAAU
		459	AGAAUCCACGUGGAAGGUCc	GACCUUCCACGUGGAUUC
		1012	UGAUGUCGCGGUAUACCACg	GUGGUAUACCGCGACAUC
PLD3	NM_012268	706	UCAUUGUUGGUGAGGGUCCa	GGACCCUCACCAACAAUG
		512	AAAGAGAUGCAAGUCGCCGt	CGGCGACUUGCAUCUCUU
		1279	ACGUAGAUGAAACUCCGGGc	CCCGGAGUUUCAUCUACG
		1051	AAGAUCUUGGUCAGGUCUCg	GAGACCUGACCAAGAUCU
LUC[a]	M15077	siLUC	AUUAAGACGACUCGAAAUCtt	GAUUUCGAGUCGUCUUAA

[a] A non-silencing control siRNA

11. Lentivirus-mediated gene overexpression (III)

A lentiviral plasmid was used for gene transfer to create cell lines with stable *MED29* overexpression. A *MED29* cDNA clone was obtained in a pT-REx-DEST31 plasmid (Invitrogen), grown in TOP10 E. coli cells (Invitrogen), and purified by use of a QIAfilter Plasmid Maxi Kit (Qiagen, Valencia, CA). The *MED29* insert was isolated by PCR amplification using two pairs of restriction primers that produced 863-bp (MED29-1) and 675-bp (MED29-2) PCR products. Both PCR products were confirmed to contain the entire *MED29* coding region by sequence analysis. BamHI and NedI restriction sites were used to clone the inserts into the pWPI lentiviral vector. pWPI with no insert was used as a control. pWPI was co-transfected with Delta 8.9 and VSVG plasmids into HEK 293T/17 cells using $CaCl_2$. The viral supernatant was harvested at 48 h after transfection, filtered, and concentrated by centrifugation. The viral concentrate was diluted in polybrene to infect NIH/3T3, Hs 700T, and MIA PaCa-2 cells. A successful transduction was evaluated by visualizing GFP (encoded by the pWPI vector), and MED29 expression was confirmed by qRT-PCR and western blot analysis.

12. Animals and tumor models (III)

Six-week-old male athymic nu/nu mice (Harlan, the Netherlands) were used to follow tumor growth *in vivo* (n=40). MED29-expressing Hs 700T and MIA PaCa-2 cells and their respective empty vector (mock) control cells were inoculated into the flanks of the mice subcutaneously, and tumor growth was followed for up to seven (Hs 700T) or eight (MIA PaCa-2) weeks. Animal welfare was monitored daily for clinical signs, and tumor measurements were performed twice per week. Tumor volume was calculated according to the formula V=(π/6)(d1xd2)3/2, where d1 and d2 are perpendicular tumor diameters. After sacrifice, the overlaying skin was removed, and the tumors were photographed. The final size of the tumors was measured with a caliper, and the volume was calculated as the length × width × depth × π/6 (Janik et al., 1975). The animal experiments were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Statutes 1076/85 § and 1360/90 of The

Animal Protection Law in Finland, and EU Directive 86/609. The experimental procedures were reviewed by the local Ethics Committee on Animal Experimentation at the University of Turku and approved by the local Provincial State Office of Western Finland.

13. Functional assays

12.1 Cell proliferation and serum starvation (III)

Cell proliferation and serum starvation assays were performed in 24-well plates and the cells were counted at 24-120 h after plating using the Coulter Counter (Beckman Coulter, Fullerton, CA). In serum starvation experiments, growth medium containing 1% FBS was used. In synchronized growth experiments, cells were grown in serum-free medium for 72 h, and their accumulation to the G1-phase of the cell cycle was confirmed by flow cytometry. Thereafter, the cells were grown in normal growth medium, and their growth was monitored for 96 h as indicated above. Each assay consisted of six replicates and was repeated at least twice.

12.2 Cell cycle analysis (I)

For the cell cycle analysis, cells were trypsinized and suspended in hypotonic staining buffer (0.1 mg/mL sodium citrate tribasic dehydrate, 0.03% Triton X-100, 50 μg/mL propidium iodide, 2 μg/mL RNase A), and the amount of incorporated propidium iodide was determined by flow cytometry (Coulter EPICS XL-MCL, Beckman Coulter, Inc., Palo Alto, CA). Cell cycle distribution was analyzed using the Cylchred program. Each experiment was done in three replicates and repeated twice.

12.3 Apoptosis assay (I)

An Annexin V FITC Apoptosis Detection Kit (Calbiochem, La Jolla, CA) was used to detect apoptotic cells by flow cytometry (Beckman Coulter). EXPO32 ADC

version 1.2 analysis software (Beckman Coulter) was applied for the analysis. All experiments were performed in three replicates and repeated twice.

12.4 Cell migration and invasion (III)

Cell culture migration chambers (8.0 μm pore size, BD Biosciences, Bedford, MA) and equivalent matrigel-coated invasion chambers (BD Biosciences) were used to study cell migration and invasion, respectively. For both assays, 5 x 10^4 cells were seeded in the upper chamber while the lower chamber was filled with culture medium. After 22h post-plating, the cells that had migrated or invaded through the pores were fixed with methanol and stained with toluidine blue. Aperio ScanScope® XT (software version 9; Aperio Technologies, USA) was used to capture images of each membrane. The total area of migrated and invaded cells on a single membrane was determined from four images quantified by use of ImageJ software (http://rsb.info.nih.gov/ij/). Each assay was performed in six replicates and repeated at least twice.

12.5 Soft agar colony formation (III)

The ability of a cell to form colonies, which is characteristic to tumor cells, was studied by growing cells on soft agar. Cells (3-10 x 10^5 per well) were grown in 0.3% agarose on a 6-well plate for 14 days. Images (six images per well) were captured with an Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan) using the Capture Pro 6.0 program. The number and total area covered by colonies were quantified using ImageJ software.

14. Microarrays (III)

12.1 Microarray gene expression

Total RNA was extracted from MED29-overexpressing MIA PaCa-2 and Hs 700T cells and their respective empty vector (mock)-transfected control cells by use of the

RNeasy Mini kit (Qiagen). The quantity and integrity of the RNA samples was assessed with a 2100 Bioanalyzer (Agilent, Santa Clara, CA). Sample (MED29) RNA was labeled with Cy5 and mock RNA with Cy3. The RNA pools were co-hybridized onto 44K whole human genome oligonucleotide microarrays (Agilent) according to the manufacturer's instructions. A total of six arrays (two biological replicates corresponding to the two ME29 constructs and three technical replicates) were prepared per cell line, and they were scanned by an Agilent microarray scanner.

12.2 Microarray data analysis

For the analysis, microarray images were transformed into spot intensity data using Agilent Feature Extraction software (version 10.5.1.1). The Limma package for the Bioconductor (Gentleman et al., 2004; Smyth, 2005) was used for preprocessing and analysis of the data. Preprocessing filtered out the spots flagged by the software as control spots, saturated, non-uniformity outliers or population outliers. Background correction was performed by the normexp method with an offset of 50 (Ritchie et al., 2007) and normalization by the LOESS normalization method (Smyth & Speed, 2003). The log ratio of normalized red (overexpression) and green (mock control) intensity values was used as the representative value for the given probe. Averages were taken for replicates representing the same mRNA. Differentially expressed genes were identified by means of empirical Bayes with Benjamini-Hochberg adjustment for P-values (Benjamini & Hochberg, 1995; Smyth, 2004). A fold change of 1.5 and an adjusted P-value of 0.05 were set for thresholds for differential expression. To examine the linkages between the samples, a hierarchical clustering method with correlation distance and average linkage was conducted. The cellular networks and canonical pathways involving the differentially expressed genes were generated through the use of Ingenuity Pathway Analysis (Ingenuity), and the enriched gene ontologies among the differentially expressed genes were obtained using DAVID bioinformatics resources (Dennis et al., 2003; Huang et al., 2009).

15. Human cell cycle RT-PCR array (III)

Ready-to-use Profiler PCR plates (SA Biosciences, Frederick, MD) were used to validate the microarray data and the effects on MED29 expression on cell cycle regulation. The plates contained 84 cell cycle-related genes, five housekeeping genes, three RT controls, three PCR controls, and a genomic DNA control. Total RNA from the MED29-expressing and the mock Hs 700T and MIA PaCa-2 cells and mice xenografts was extracted as described above with an additional DNase treatment step. The RNA content was measured with the Bioanalyzer (Agilent) or NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). One microgram of total RNA was transcribed into cDNA and PCR-amplified according to the manufacturer's instructions. PCR amplification and detection were performed using Bio-Rad CFX96 equipment (Bio-Rad, Hercules, CA), and the data were analyzed with the PCR Array Data Analysis protocol (SA Biosciences). The threshold cycle (Ct) values for each gene were normalized using the average of the five housekeeping genes on the same array.

16. Statistical analyses (I-III)

Mann–Whitney test was utilized to compare the medians of the test and control groups in all functional assays (I, III). Statistical analyses for the TMA (II) were performed using JMP™ software (SAS Institute Inc. Cary, NC, USA). All tested P-values were two sided, and a P-value of less than 0.05 was considered significant. The Pearson Chi-square test was used to assess the relationship between 19q13 copy number changes and the clinicopathologic parameters (tumor stage, nodal stage, and tumor grade). Association of 19q13 copy number changes with cancer-specific survival was studied using the Kaplan-Meier method, and the log rank test was applied to test the significance between stratified groups. The statistical methods used in the microarray data analysis are described above (12.2. Microarray data analysis).

RESULTS

1. Characterization of the 19q13 amplicon and identification of putative target genes (I)

A previous genome-wide aCGH profiling identified a ~2.9-Mb region of amplification in pancreatic cancer (Mahlamäki et al., 2004) that was here systematically characterized in 16 pancreatic cancer cell lines using fluorescence *in situ* hybridization (FISH). Fifteen evenly distributed BAC clones across the amplicon were selected for DNA copy number analysis, which revealed copy number increases (relative copy number > 2) in three cell lines: PANC-1, SU.86.86, and HPAC. Up to 20-fold amplification was detected in PANC-1 cells, whereas SU.86.86 and HPAC cells harbored lower-level gains (up to 5.3- and 2.6-fold). The copy number profile of PANC-1 cells was used to define the minimal amplified region, and in this way, the original amplicon of approximately 2.9 Mb could be outlined to 1.1 Mb. Within this 1.1 Mb minimal amplified region, defined by six overlapping BAC clones from RP11-67A5 to CTC-425O23, a 660-kb core region (from RP11-67A5 to CTC-488F21) of high-level (over 10-fold) amplification was identified.

Next, the effects of amplification on gene expression were explored. With the help of public genomic databases (UCSC Genome Browser http://www.genome.ucsc.adu and NCBI Genome http://www.ncbi.nlm.nih.gov), 39 transcripts were identified within the 1.1 Mb amplicon. Of these transcripts, 27 were known genes and 12 were hypothetical or predicted proteins (Table 5). Careful examination, including bioinformatics and gene expression analyses of PANC-1 cells, revealed appropriate genes for comprehensive quantitative RT-PCR analysis. Sixteen genes were excluded as putative candidates according the following criteria: they represented (a) obvious pseudogenes, (b) predicted transcripts with no mRNA or expressed sequence tag (EST) evidence in the databases, or (c) transcripts with no

or low expression in PANC-1 cells. The last criterion was based on the concept that a putative target gene should be expressed in cells with high-level amplification.

Table 5. *List of the 39 genes within the 1.1Mb amplicon at 19q13.*

Gene	Description
IL29	Interleukin 29
LRFN1	Leucine rich repeat and fibronectin type III domain containing 1
GMFG	Glia maturation factor gamma
SAMD4B	Sterile alpha motif domain containing 4B
PAF1	RNA polymerase II associated factor, homolog (S. cerevisiae)
MED29	Mediator subunit 29
ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)
PLEKHG2	Pleckstrin homology domain containing family G member 2
RPS16	Ribosomal protein S16
SUPT5H	Suppressor of Ty 5 homolog (S. cerevisiae)
TIMM50	Translocase of inner mitochondrial membrane 50 homolog (yeast)
DLL3	Delta-like 3 (Drosophila)
SELV	Selenoprotein V
EID-3	EID-2-like inhibitor of differentiation-3
CRI2	CREBBP/EP300 inhibitor 2
LOC390930	Similar to Eosinophil lysophospholipase (Charcot-Leyden crystal protein)
LGALS13	Lectin, galactoside-binding, soluble, 13 (galectin 13)
LOC441850	Similar to Eosinophil lysophospholipase (Charcot-Leyden crystal protein)
LOC148003	Similar to Placental tissue protein 13 (Placenta protein 13) (Galectin-13)
LOC400696	Eosinophil lysophospholipase-like
LGALS14	Lectin, galactoside-binding, soluble, 14
CLC	Charcot-Leyden crystal protein
LOC342900	Hypothetical protein LOC342900
DYRK1B	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B
FBL	Fibrillarin
FCGBP	Fc fragment of IgG binding protein
LOC440525	Proline rich 13 pseudogene
PSMC4	Proteasome (prosome, macropain) 26S subunit, ATPase, 4
ZNF546	Zinc finger protein 546
LOC390933	Similar to hypothetical protein
LOC163131	Hypothetical protein LOC163131
LOC284323	Hypothetical protein LOC284323
MAP3K10	Mitogen-activated protein kinase kinase kinase 10
TTC9B	Tetratricopeptide repeat domain 9B
FLJ13265	Hypothetical protein FLJ13265
LOC440526	Hypothetical protein LOC440526
AKT2	v-akt murine thymoma viral oncogene homolog 2
FLJ36888	Hypothetical protein FLJ36888
PLD3	Phospholipase D family, member 3

The remaining 23 genes were included in the quantitative RT-PCR analysis of 16 pancreatic cancer cell lines. Most of the genes (22 of the 23) were overexpressed in PANC-1 cells compared to other pancreatic cancer cell lines and normal pancreas. Only PLD3, which is located at the border of the amplicon, was not overexpressed in PANC-1 cells. Gene expression levels in HPAC and SU.86.86 cells showed more variability between individual genes and were thus utilized to distinguish differentially expressed genes between amplified and nonamplified cell lines. Six known genes (*GMFG*, *SAMD4B*, *IXL (MED29)*, *SUPT5H*, *PSMC4*, and *MAP3K10*) and one hypothetical protein (*LOC284323*) were consistently overexpressed in all three amplified cell lines. Of these, *GMFG*, *SAMD4B*, *MED29*, and *SUPT5H* were located in the 660 kb amplicon, making them more likely targets than *PSMC4*, *MAP3K10*, and *LOC284323,* which were located outside of this core

region. Interestingly, high-level expression of *MED29* was almost exclusively detected in the amplified cells, indicating a clear distinction between the amplified and nonamplified cells (Figure 6). In contrast, *GMFG* and *MAP3K10* showed high expression in additional non-amplified cells, indicating additional mechanisms that give rise to increased expression.

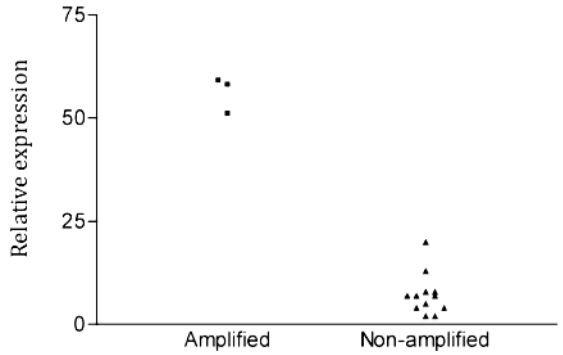

Figure 6. *Relative MED29 gene expression in the 19q13 amplified vs. non-amplified pancreatic cancer cell lines.*

To identify functionally important candidates, a targeted high-throughput RNAi screen across the 1.1 Mb amplicon was applied (Ngo et al., 2006). Four siRNAs were designed for each of the 19 known genes (only those that were known genes at the time of selection and were expressed in PANC-1 cells were included), and their effect on cell viability was examined in PANC-1 and MIA PaCa-2 cells at 96 h after transfection. Cell viability was significantly reduced (i.e., viability less than 50%) through downregulation of *MED29* in the amplified PANC-1 cells but not in the nonamplified MIA PaCa-2 cells. This effect was shown with two independent siRNAs in three repeated experiments (Table 6). Reduced cell viability (i.e., viability less than 50%) was also observed after *LRFN1* and *PLEKHG2* silencing but only with a single siRNA in repeated experiments (Table 6). Down-regulation of *GMFG* and *SUPT5H* showed a slight reduction in cell viability but no other examined genes had a statistically significant effect on cell viability when silenced.

***Table 6**. Results from the cell viability screen.*

siRNA	PANC-1 (I)	PANC-1 (II)	PANC-1 (III)	MIAPaCa-2 (I)	MIAPaCa-2 (II)
AKT2 1012	0.83	0.84	0.93	0.75	0.56
AKT2 1511	1.07	1.05	1.01	1.10	0.65
AKT2 459	1.10	1.06	0.96	1.09	0.57
CRI2 621	0.69	0.79	0.98	0.95	0.94
CRI2 685	0.61	0.69	1.02	0.88	0.99
CRI2 780	0.71	0.78	1.02	0.69	1.01
CRI2 796	1.13	1.10	1.00	1.14	1.22
DLL3 117	0.86	0.95	0.96	1.14	1.16
DLL3 1862	0.99	1.02	0.92	1.05	1.16
DLL3 1968	0.56	0.55	0.41	0.71	0.76
DLL3 2302	0.52	0.49	0.83	0.85	0.93
DYRK1B 1345	0.76	0.80	0.98	0.85	1.01
DYRK1B 1607	1.10	1.10	0.96	1.14	1.18
DYRK1B 599	0.67	0.69	0.43	0.86	0.83
DYRK1B 778	0.80	0.77	0.85	0.86	0.63
FBL 644	0.51	0.42	0.47	0.23	0.41
FBL 807	1.00	0.99	0.93	1.01	1.05
FBL 819	0.77	0.76	0.84	0.67	0.83
FBL 948	0.96	0.91	0.89	0.87	1.01
FCGBP 12000	0.80	0.87	0.95	1.04	0.75
FCGBP 5974	1.08	1.04	0.99	1.08	0.98
FCGBP 6395	0.65	0.70	0.66	0.74	0.67
FCGBP 780	0.80	0.73	0.90	0.75	0.76
LRFN1 188	0.45	0.57	0.71	0.94	1.00
LRFN1 2069	0.34	0.63	0.83	0.97	1.04
LRFN1 754	0.57	0.68	0.84	0.94	1.06
LRFN1 762	0.31	0.36	0.45	0.81	0.94
MED29 144	**0.16**	**0.37**	**0.62**	**1.04**	**1.13**
MED29 261	**0.81**	**1.01**	**0.91**	**1.05**	**1.14**
MED29 264	**0.71**	**0.80**	**0.85**	**0.94**	**0.99**
MED29 584	**0.30**	**0.28**	**0.13**	**0.92**	**1.11**
GMFG 198	0.65	0.69	0.91	0.93	1.06
GMFG 207	0.67	0.83	0.92	0.97	1.08
GMFG 243	0.46	0.70	0.81	1.03	1.09
GMFG 27	0.51	0.58	0.58	0.99	1.03
MAP3K10 1326	1.05	1.10	0.97	1.00	0.77
MAP3K10 1351	1.12	1.19	1.05	1.13	0.94
MAP3K10 1719	0.85	0.98	1.03	0.89	0.79
MAP3K10 2401	1.19	1.16	1.02	1.14	1.05
MAP3K10 631	1.14	1.12	1.00	1.11	0.66
PAF1 1051	0.58	0.87	0.73	1.13	1.20
PAF1 267	0.96	1.06	0.82	1.12	1.19
PAF1 555	0.79	0.70	0.91	1.02	1.08
PAF1 587	0.60	0.97	0.71	1.08	1.16
PLD3 1051	1.14	1.08	1.00	1.14	0.89
PLD3 1279	1.13	1.15	1.03	1.14	0.85
PLD3 512	1.13	1.07	1.01	0.95	0.84
PLD3 706	0.81	1.01	1.00	0.92	0.97
PLEKHG2 1677	0.30	0.33	0.58	0.86	0.88
PLEKHG2 1774	0.65	0.83	0.84	1.10	1.17
PLEKHG2 3219	0.55	0.70	0.85	1.15	1.13
PLEKHG2 3463	0.55	0.65	0.61	1.12	1.16
PSMC4 200	0.73	0.74	0.69	0.34	0.58
PSMC4 260	0.72	0.83	0.87	0.53	0.50
PSMC4 596	0.96	0.90	0.83	0.64	0.78
PSMC4 752	1.04	0.97	0.94	1.02	0.92
RPS16 161	0.75	0.75	0.46	0.85	1.11
RPS16 308	0.73	0.74	0.47	0.80	1.05
RPS16 313	0.82	0.89	0.43	0.73	1.02
RPS16 360	0.55	0.72	0.57	0.82	1.04
SUPT5H 2304	0.57	0.68	0.36	1.02	1.08
SUPT5H 2694	0.74	0.87	0.56	1.02	0.98
SUPT5H 3363	0.55	0.64	0.52	0.83	0.77
SUPT5H 525	0.61	0.83	0.72	1.04	1.02
TIMM50 1099	0.50	0.82	0.83	0.99	1.10
TIMM50 1123	0.57	0.69	0.75	0.89	1.19
TIMM50 676	0.68	0.87	0.89	1.00	1.20
TIMM50 964	0.57	0.58	0.47	0.57	0.98
ZFP36 1524	0.75	0.80	0.97	1.04	1.10
ZFP36 376	0.86	1.01	0.95	1.15	1.19
ZFP36 488	0.68	0.80	0.67	1.12	0.98
ZFP36 890	1.00	1.04	0.85	1.20	1.19
ZNF546 192	0.99	1.04	1.00	1.08	0.97
ZNF546 2279	0.89	0.90	0.98	0.77	0.83
ZNF546 2656	1.00	0.95	0.94	0.96	0.50
ZNF546 743	1.00	1.04	0.96	0.90	0.60

Each experiment lists the average viability of siRNA-treated cells relative to non-silencing siRNA-treated cells. Viability less than 0.50 in repeated experiments was considered as a significant change.

2. Clinical significance of the 19q13 amplification in pancreatic cancer (I, II)

After identification of the 19q13 amplification in pancreatic cancer cell lines, we investigated whether the same change was present in cancers of other tissues, and most importantly, in primary pancreatic tumors. The amplification status of the core region of the 19q13 amplicon was evaluated in a broad collection of 120 cell lines derived from multiple tumor types, in a panel of 31 primary tumors of the pancreas, and in a large panel of over 500 pancreatic tumor specimens.

In the first experiment we determined the amplification status of the 19q13 core amplicon in 120 cancer cell lines representing nearly 20 different tumor types. Ten (9.3%) of the 107 cell lines with successful hybridizations had an increased copy number. Six of these were amplifications (relative copy number over 2), and four were gains (relative copy number >1.5 but <2). Amplifications were detected in one (OVCAR-3) of four ovarian cancer cell lines, three (RT-112, KU-19-19, CRL-7930) of six bladder cell lines, one (SW-48) of twelve colorectal cell lines, and one (ONCO-DG-1) of four thyroid carcinoma cell lines.

In the smaller set of primary tumors, 19q13 amplification was observed in three (9.7%) of the 31 analyzable cases. All of these amplified tumors were moderately to poorly differentiated (grade 2 or 3) pancreatic adenocarcinomas with lymph node metastases. In addition, two of the three amplified cases had extensions to peripancreatic soft tissue with perineural invasion. The metastatic status of the third amplified case was not available. Although the sample set was small, it provided a promising starting point with an amplification frequency of almost 10% and an indication of possible association of amplification to higher tumor grade.

The frequency of the 19q13 copy number changes was then explored in 357 primary pancreatic tumors, 151 metastatic lesions, and 24 local recurrences. Copy number increases were detected in 12.2% of the 303 analyzable cases. Changes in copy number were most frequent in ductal adenocarcinomas and pancreatic endocrine tumors (Table 7). One of 15 IPMNs showed increased copy number. Amplification typically manifested as a tight cluster of signals with an average 3.4-fold copy number increase, but a few cases with up to 10-fold amplification were also observed. The average copy number of the 19q13 locus per cell in polysomic samples was between five and ten, but it ranged up to twenty. Copy number

increases were detected in 7 (5.8%) of the 121 successfully hybridized metastases, of which 3 were amplifications and 4 were gains. Copy number data from the primary tumors matched well with the metastases, showing an overall concordance of 99% (90/91). Copy number changes were not detected in the local recurrences (total of 18 analyzable cases).

***Table** 7. Primary pancreatic cancer subtypes and their copy number status.*

	n	analyzable	normal	CNI
All samples	357	303	87.8	12.2
Ductal adenocarcinomas	213	197	86.3	13.7
Intraductal papillary mucinous	33	15	93.3	6.7
Endocrine tumors	40	35	88.6	11.4
Cystic/benign tumors	15	6	100	0.0
Cystic/malignant tumors	1	1	100	0.0
Acinar cell cancers	1	0	-	-
Adenocarcinomas of the ampulla of	54	49	89.8	10.2
Metastases	151	121	95.0	5.0
Lymph node metastases	129	100	96.0	4.0
Distant metastases	22	21	90.4	9.6
Local recurrences	24	18	100	0.0

CNI = copy number increases (amplifications, polysomy, gains)

To evaluate the possible clinical importance of the 19q13 amplification, we examined associations between the copy number status and clinicopathological characteristics among the ductal adenocarcinomas, the most common histological subtype of pancreatic cancer. To this end, tumors confined to the pancreas (pT1 and pT2) were compared to tumors that had spread beyond the pancreas (pT3 and pT4). Likewise, moderately and well-differentiated tumors (G1 and G2) were compared to poorly differentiated tumors (G3). Copy number increases (including gains, amplifications, and polysomy) showed association with both tumor grade and stage (P=0.044 and P=0.025, respectively). In addition, the frequency of copy number changes increased from low- and moderate- (G1-G2) to high-grade (G3) tumors and from early- (pT1-pT2) to late- (pT3-pT4) stage tumors. Copy number changes were not detected in any of the low-grade (G1) tumors, whereas 11% of grade 2 tumors and 16.8% of grade 3 tumors harbored an increased copy number. High-level amplifications were exclusively observed in grade 3 tumors (Table 8).

***Table 8**. Relationship between the 19q13 copy number changes and the cancer phenotype in pancreatic ductal adenocarcinomas.*

		n	normal (%)	CNI (%)	P
pT stage	pT1	6	83.3	16.7	0.025
	pT2	53	92.5	7.5	
	pT3	125	84.8	15.2	
	pT4	9	66.7	33.3	
pN stage	pN0	68	83.8	16.2	0.826
	pN1	124	87.1	12.9	
Grade	G1	8	100	0.0	0.044
	G2	91	89.0	11.0	
	G3	95	83.2	16.8	

CNI = copy number increases (amplifications, polysomy, gains)

At last, we performed Kaplan-Meier survival analysis on the ductal adenocarcinomas, which showed slightly worse survival in patients having high level copy number changes (amplifications and polysomy) compared to those not having 19q13 copy number changes, but the difference was not statistically significant. However, the average survival time for the patient group without a 19q13 change was 26 months, whereas patients with amplification or polysomy survived an average of only 16 and 17 months, respectively. Taken together, association of the 19q13 amplification with tumor stage and grade and shorter survival time among patients with the aforementioned amplification indicates a trend towards a worse prognosis.

3. Functional characterization of MED29 (I, III)

After fine-mapping of the 19q13 amplicon, comprehensive gene expression analysis and subsequent first-line functional screening, we aimed to perform functional validation of the candidate target genes. To select the most promising target gene for further functional characterization, we combined the results of the gene expression survey and the RNAi-based loss-of-function screen. MED29 was the most attractive target because it was located within the 660 kb amplicon and was implicated in both screens. MED29 was downregulated by siRNA transfection of amplified PANC-1 cells, and the effects of silencing were explored in multiple functional assays, including proliferation, cell cycle, apoptosis, soft agar colony formation, migration,

and invasion assays. Cell cycle and apoptosis were assessed at 48 h after siRNA transfection by flow cytometry. An increased fraction of G0-G1 phase cells after MED29 siRNA (66%) transfection compared with nontransfected cells (37%) or cells transfected with a control siRNA (39%) was detected in cell cycle analysis. Similarly, the percentage of apoptotic cells increased after MED29 siRNA transfection (8%) compared to cells transfected with control siRNA (1.6%) or untreated cells (2.5%). However, similar phenotypic changes were not observed in non-amplified MIA PaCa-2 cells when transfected with MED29 siRNAs. In addition, we observed an average of 18% reduction in cell number at 24-96 h after MED29 siRNA transfection in PANC-1 cells. Similarly, a 26% growth reduction was observed in SU.86.86 cells, another MED29-amplified pancreatic cancer cell line, following MED29 silencing. Colony formation was assessed by growing cells in soft agar. MED29-silenced PANC-1 cells were found to grow more slowly compared to control cells. Both the size and the number of colonies were smaller in the MED29-silenced cells. Furthermore, cell migration (47% reduction) and invasion (43% reduction) were also considerably compromised after MED29 silencing. In parallel with all siRNA experiments, sufficient gene silencing was verified by qRT-PCR. MED29 protein levels were likewise notably reduced by siRNA transfection. Taken together, MED29 silencing in pancreatic cancer cells with high endogenous expression seems to diminish several cancer-associated phenotypic characteristics.

To study the effects of forced MED29 expression in cells with no or low endogenous expression, we used a lentiviral-based method to generate cells with stable MED29 overexpression. MED29 was transduced into NIH/3T3, Hs 700T, and MIA PaCa-2 cells. NIH/3T3 is a mouse fibroblast cell line, and Hs 700T and MIA PaCa-2 are pancreatic cancer cell lines. Significant increases in MED29 mRNA and protein expression were achieved in all cell lines compared to their empty vector controls. Decreased cell number was observed in MED29-transduced NIH/3T3 cells when followed for four days. Similarly, up to a 30% growth reduction compared to the mock cells was observed for MIAPaCa2/MED29 cells when synchronized to G1-phase of the cell cycle prior to growth analysis. Unfortunately, we were unable to synchronize the Hs700T/MED29 cells and did not see any consistent growth affect in these cells *in vitro*.

We continued to explore the possible role of MED29 in *in vivo* tumor formation. MED29-transduced Hs 700T and MIAPaCa-2 cells, and their corresponding empty vector cells were subcutaneously inoculated into nude mice and allowed to grow for seven (Hs 700T) or eight (MIA PaCa-2) weeks. A striking difference in tumor growth and incidence was observed between MIAPaCa2/MED29 and MIAPaCa2/mock mice (Figure 7). The MIAPaCa2/mock tumors showed a clear exponential growth pattern from day 20 of the experiment onward, whereas MIAPaCa2/MED29 tumors remained small throughout the entire observation period. At the end of the experiment, the average volume of the MIAPaCa2/mock tumors was 13-fold greater than that of MIAPaCa2/MED29 tumors. A comparable but less dramatic decrease in tumor growth was also seen in the Hs700T/MED29 mice. The average size of the Hs700T/mock tumors at the end of the study was 1.3-fold compared to Hs700T/MED29 tumors. All tumors displayed a vascularized morphology, implying sustained functional angiogenesis. MED29 mRNA levels were verified in the MED29 tumors after the experiment. These data clearly indicate that MED29 expression leads to tumor suppression *in vivo*.

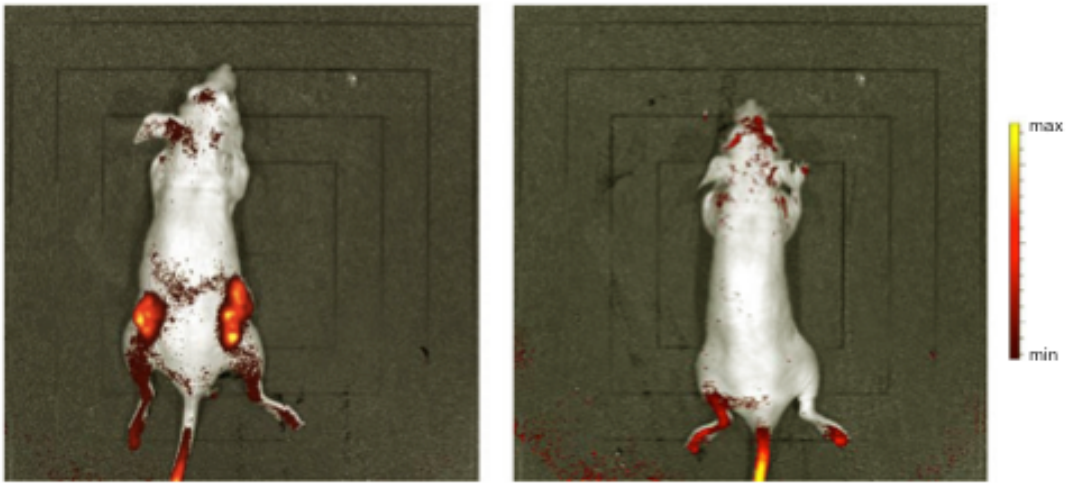

Figure 7. *MED29 expression inhibits tumor growth in vivo. Fluorescence-imaged MIAPaCa2/mock (left) and MIAPaCa2/MED29 (right) mice.*

4. Transcriptional effects of MED29 expression (III)

To conceptualize the mechanism of MED29 action and to better understand the causes of the observed *in vitro* and *in vivo* growth differences, a genome-wide microarray gene expression analysis between the MED29- and mock-transduced Hs 700T and MIA PaCa-2 cells was performed. A total of 463 differentially expressed genes (≥1.5-fold change with adjusted P-value <0.05) were recognized between the MED29-overexpressing and the corresponding mock cells in these two pancreatic cancer cell lines. On the whole, downregulation was more prevalent than upregulation (65% vs. 35%) among the differentially expressed genes, and the fraction of differentially expressed genes was greater in MIA PaCa-2 than Hs 700T cells. Hierarchical clustering of the differentially expressed genes identified common up- and down-regulated gene clusters between MIAPaCa2/MED29 and Hs700T/MED29 cells. Biological and technical replicates clustered together as expected.

Gene ontology data for these 463 differentially expressed genes revealed several key biological processes implicated in the cell cycle, cell division, mitosis, and chromosome segregation to be enriched in MED29-transduced cells (Figure 8). Concomitant cellular component analysis showed enrichment in condensed chromosomes, kinetochore, spindle, and microtubule cytoskeleton, indicating a central role in cell division. Many well-known cell cycle regulatory genes, such as *CCNA2*, *CCNB2*, *CDK1*, *GTSE1*, and *CDKN2B,* were identified among the enriched categories. Cell cycle-promoting genes (*CCNA2*, *CCNB2*, *CDK1*, and *GTSE1*) were downregulated in MED29-expressing cells, whereas *CDKN2B*, an inhibitory regulator of the cell cycle, was upregulated.

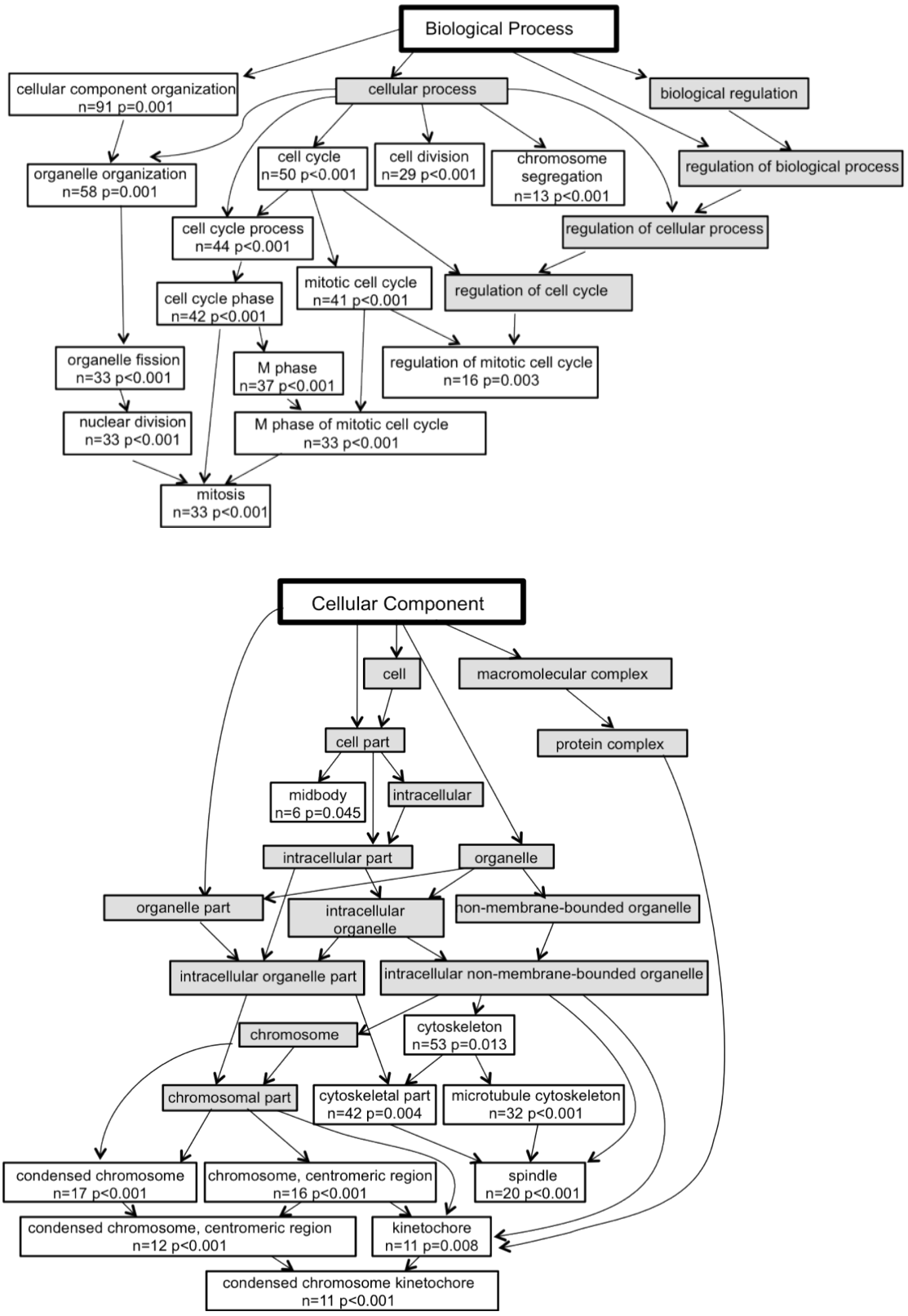

Figure 8. *Enriched gene ontologies among the differentially expressed genes (MED29 vs. mock cells) involving (a) biological processes and (b) cellular components are illustrated here. The main category is shown at the top of the figure and white boxes represent enriched gene categories. The number of enriched genes (n) in each category is shown in the box with the adjusted P-value for enrichment.*

To gain insight into the functional role of the genes with altered expression after MED29 overexpression, Ingenuity Pathway Analysis was performed on the differentially expressed genes. This pathway analysis identified a network that associates with the cell cycle, cancer, and genetic disorders. The highest enrichment among molecular and cellular functions included the cell cycle, cell death, and cellular growth and proliferation. The most affected canonical pathways involved the cell cycle. To conclude, enrichment of cell cycle-associated cellular networks and functions was observed in the pathway analysis of differentially expressed genes.

A qRT-PCR-based method was applied to verify the microarray data and to systematically characterize the role of cell cycle regulators as transcriptional targets of MED29. Gene expression levels of 84 known cell cycle genes were simultaneously validated in MIAPaCa2/MED29 and Hs700T/MED29 cells by qRT-PCR. The obtained results correlated well with the microarray data, identifying the same cell cycle regulators (e.g., *BAX*, *CCNF*, *CDC34*, *CDK2*, *CDK6*, *CDKN1A*, *GADD45*, and *GTF2H1*) as being differentially expressed. In addition, we explored whether alterations in these cell cycle regulators were present in the xenograft tumors derived from these cell lines. A total of 12 cell cycle genes were found to be differentially expressed (fold change greater than 1.5) in MIAPaCa2/MED29 xenografts compared to MIAPaCa2/mock xenografts, of which 92% (11 out of 12) were downregulated. Common cell cycle-promoting genes, including *CCND2*, *CDC16*, *CDK6*, *HERC5*, and *RBBP8*, were downregulated in both MIAPaCa2/MED29 xenografts and parental MIAPaCa2/MED29 cells. *CDKN2B*, a negative regulator of the cell cycle, was the only upregulated gene in the MIAPaCa2/MED29 xenografts, also showing elevated expression in the parental MIAPaCa2/MED29 cells. In Hs 700T cells and xenografts, we did not notice any dramatic differences in cell cycle gene expression levels between the MED29 and mock cells. Only five differentially expressed genes were identified in Hs700T/MED29 tumors compared to mock tumors. Of these, *ANAPC4*, *ATR*, *CDK4*, and *SUMO1* were downregulated, whereas *HERC5* was upregulated. To summarize, this PCR-based method enabled us to validate the microarray data and highlighted some MED29-induced changes that are essential for the expression of cell cycle-related genes.

DISCUSSION

1. Amplification of the 19q13 locus associates with tumor grade and stage (I, II)

Gene amplification is a typical mechanism employed by solid tumors to activate growth-promoting oncogenes (Albertson et al., 2003) and a frequent phenomenon in pancreatic cancer (Hansel et al., 2003). Multiple amplified regions have been documented in carcinomas of the pancreas (Harada et al., 2008; Jones et al., 2008), but few target genes have been functionally characterized (Santarius et al., 2010), and minimal progress has been made in early diagnosis or treatment (Hidalgo, 2010). Detailed analysis of amplified regions and extensive functional validation may bring out novel targets for diagnostic and therapeutic purposes. To this end, we performed a comprehensive copy number analysis of the amplified 19q13 locus in pancreatic cancer cell lines, delineating the amplicon to 1.1 Mb, with a 660-kb core region of extremely high amplification. This 660-kb core region was of particular interest because amplification target genes are likely to be located at or near the center of the amplification maximum (Albertson, 2006).

In addition to pancreatic cancer, 19q13 amplification has been reported in e.g., ovarian (Cheng et al., 1992; Bellacosa et al., 1995; Thompson et al., 1996; Tang et al., 2002), breast (Bellacosa et al., 1995; Yu et al., 2009), colorectal (Knösel et al., 2004), and urinary bladder (Richter et al., 2000) cancers. Nevertheless, this study was the first to characterize this specific core region of amplification in a systematic manner across a wide range of tumor types. Our cell line panel contained over 100 samples derived from nearly 20 tumor types. Amplification of the 19q13 core region was identified in ovarian, colorectal, urinary bladder, and thyroid cancer cell lines, confirming previous findings for these tissues (Cheng et al., 1992; Bellacosa et al., 1995; Thompson et al., 1996; Richter et al., 2000; Knösel et al., 2004; Tang et al., 2002) with an exception of thyroid cancer, in which the 19q13 amplification had not been identified previously. Furthermore, these data provide valuable information on

suitable cell lines for functional analysis of putative 19q13 target genes for multiple tumor types.

The presence of this core region of amplification was analyzed in primary pancreatic tumors using FISH to TMAs. Evaluation was first accomplished in a small set of 31 pancreatic tumors, which showed an amplification frequency of approximately 10%. The amplified cases were moderately to poorly differentiated tumors with nodal metastases, two of which had additional local metastases indicative of advanced disease. A similar copy number analysis was carried out in an extensive collection of over 500 pancreatic tumors to establish possible clinicopathological correlations. Copy number increases were detected in 12.2% of the samples, showing good concordance with the smaller sample set. In addition, copy numbers of primary tumors and their corresponding metastases correlated well with one another.

Gene amplification has been linked to tumor progression, drug resistance, and poor clinical outcome in multiple cancers (Seeger et al., 1985; Slamon et al., 1987; Savelyeva and Schwab, 2001). For example, amplifications of *CCND1*, *EGFR*, *ERBB2*, *MDM2*, and *MYC* are associated with high tumor grade, and amplifications of *MYC* and *ERBB2* correlate with shortened survival in breast cancer (Al-Kuraya et al., 2004). In addition, amplification of *MYCN* is a prognostic factor in neuroblastoma, and amplifications of *DHFR* and *BCR-ABL1* are associated with resistance to therapies (Albertson, 2006). We therefore explored whether the 19q13 amplification is associated with clinicopathological characteristics and patient survival in pancreatic cancer. We found that the 19q13 copy number increases were linked to both advanced tumor stage and grade. This is a novel and important finding given that only a few amplified genes have been linked to advanced disease in pancreatic cancer (Friess et al, 1995; Gansauge et al., 1997). In ovarian carcinoma, the 19q13 amplification has previously been reported to associate with less differentiated and more aggressive tumors (Bellacosa et al., 1995), supporting our finding. To further strengthen our data, we sought to examine MED29 protein expression in the same panel of samples by immunohistochemistry. Unfortunately, the custom-made antibody that we had successfully used in western blot analysis failed to show specific staining of the tissues despite numerous trials.

Kaplan-Meier survival analysis was used to evaluate the effect of the 19q13 amplification on patient survival. Although the analysis did not provide statistically

significant evidence of shorter survival, the average survival time of patients with high-level amplification was notably shorter than the survival of patients with normal copy number status. The lack of statistical power in the analysis might be a consequence of the fairly small number of tumors with copy number aberrations or it may be due to the overall dismal prognosis of pancreatic cancer. In any case, our data are concordant with the conception that high level amplifications typically have a greater impact on tumor development than low-level aberrations (Hodgson et al., 2003). Furthermore, amplification of the 19q13 locus has been previously associated with poor survival of patients with non-small cell lung cancer (Kim et al., 2005). Taken together, these studies provide a wealth of new information. A distinct amplicon core was delineated in pancreatic cancer cell lines and validated in primary pancreatic tumors, its association with high tumor grade and stage was revealed, and a tendency towards shorter patient survival was demonstrated.

2. *MED29* functions as a cancer promoting gene when endogenously amplified and overexpressed (I, III)

Comprehensive gene expression analysis across the amplified region revealed several putative target genes. Given that all overexpessed genes within an amplicon theoretically represent putative candidate genes (Albertson, 2006), we chose to perform a targeted high-throughput RNAi screen to identify functionally relevant genes (Santarius et al., 2010; Ngo et al., 2006). Combination of data from the expression analysis and the RNAi screen enabled us to systematically recognize genes with increased expression levels due to amplification and for which down-regulation resulted in phenotypic change. This strategy identified MED29 as the most promising target because it was overexpressed in the cells with amplification of the locus and showed the most dramatic reduction in cell viability in amplified PANC-1 but not in non-amplified MIA PaCa-2 cells when silenced. Silencing of other genes, including *LRFN1*, *PLEKH2G*, *SUPT5H*, and *GMFG,* decreased cell viability, but the change was not as clear or consistent as that observed for *MED29*. Based on these data, *MED29* seems to be the strongest candidate target for the 19q13 amplification, although we cannot exclude the involvement of other

overexpressed genes in the region. It is noteworthy that the RNAi screen was solely based on cell viability. Selection of another phenotypic endpoint might have yielded different results. Another issue concerning large-scale screens is that individual siRNAs can not be validated individually, meaning that specific and efficient silencing is not always obtained. To avoid biased results, four distinct siRNA molecules were used for each gene in repeated experiments.

MED29 silencing experiments revealed increased apoptosis and G0-G1 arrest in 19q13-amplified PANC-1 cells but not in the non-amplified MIA PaCa-2 cells. Similarly, reduced cell growth was obtained in *MED29*-silenced 19q13-amplified SU.86.86 cells. Soft agar colony formation, cell migration, and invasion, which are typical tumorigenic characteristics (Hanahan & Weinberg, 2000), were significantly compromised in PANC-1 cells after silencing of *MED29*. These data suggest that *MED29* provides cancer cells with multiple beneficial oncogenic properties when amplified and overexpressed. Furthermore, decreased cell growth and survival after *MED29* downregulation suggests that cells with amplified *MED29* become dependent on it, a phenomenon known as oncogene addiction (reviewed by Weinstein & Joe, 2008).

Other suggested targets for the 19q13 amplification in pancreatic cancer include *ACTN4* (Kikuchi et al., 2008), *AKT2* (Cheng et al., 1992), *DYRK1B* (Deng et al., 2006), *PAF1* (Moniaux et al., 2006), and *PAK4* (Chen et al., 2008). *AKT2* is a human homolog of the viral v-akt oncogene, which causes leukemia in mice (Staal et al., 1987). Amplification of *AKT2* has been reported in ovarian carcinoma (Cheng et al., 1992) and in other cancers (Bellacosa et al., 1995). Our data show amplification of *AKT2,* but it was not consistently overexpressed in all amplified cell lines, and the silencing of *AKT2* did not reduce cell viability. *ACTN4* encodes an actin-binding protein that is involved in cell motility and invasion and has been shown to be amplified and overexpressed in pancreatic cancer (Kikuchi et al., 2008). However, *ACTN4* was not within the minimal region delineated here and is likely located in a separate amplicon. *DYRK1B* is a serine/threonine kinase that mediates survival and is overexpressed in pancreatic cancer (Deng et al., 2006). *DYRK1B* was considerably overexpressed in two of our amplified cell lines and is a possible target in the region, although the viability screen did not support this finding. *PAF1* is a human homologue of the RNA polymerase II-associated factor that is reported to promote tumor growth (Moniaux et al., 2006), but it was only moderately expressed

in the amplified cell lines in our analysis. *PAK4* is a member of the p21-activated kinase family that was recognized as a putative 19q13 target along with *MED29* and *PAF1* in the representational oligonucleotide microarray analysis (Chen et al., 2008). Unfortunately *PAK4* was located outside of the amplified region delineated in this study and was thus not included in our expression analysis. Based on these reports, it is likely that this amplicon contains several target genes.

3. Forced MED29 expression leads to attenuated tumor growth and downregulation of cell cycle genes (III)

To gain a more thorough insight into the role of MED29 in pancreatic cancer, MED29 was overexpressed in three models: NIH/3T3, Hs 700T, and MIA PaCa-2 cells. NIH/3T3 is s mouse fibroblast cell line commonly used in gene transfer experiments because it is easy to handle and transfect. Hs 700T and MIA PaCa-2 pancreatic cancer cells served as suitable models due to their low endogenous MED29 expression levels. MED29 expression resulted in the reduced proliferation of NIH/3T3 and synchronized MIA PaCa-2 cells. This finding was unexpected because we had previously observed decreased cell growth after MED29 silencing in PANC-1 and SU.86.86 cells. However, different cell types and different genetic backgrounds may explain the opposite effects (Deer et al., 2010). Decreased cell growth in non-cancerous fibroblasts could also be explained by the induction of senescence, similar to what has been reported with KRAS and RAF oncogenes (Serrano et al., 1997; Zhu et al., 1998). In contrast, MED29 expression had no consistent effect on Hs 700T cell growth in vitro, which might be a consequence of differences in MED29 expression levels between the cells or the fact that we could not synchronize cell growth as we did with MIA PaCa-2 cells.

Most importantly, MED29 led to significantly decreased tumor formation in mice xenografts of MIA PaCa-2 and Hs 700T cells. Mock-transfected xenografts formed large tumors, whereas MED29-transfected xenografts developed much more slowly, forming considerably smaller tumors. MED29-expressing MIA PaCa-2 xenografts showed greatly reduced tumor incidence and a minimal growth increase during the observation period in contrast to MIA PaCa-2 mock tumors, which

exhibited exponential growth and a 100% tumor incidence. The effects of MED29 on Hs 700T xenografts were similar to those on MIA PaCa-2 xenografts, but they were not as dramatic. These *in vivo* data firmly suggest that MED29 also possesses some tumor-suppressive properties.

The opposing phenotypic characteristics revealed by MED29 silencing and overexpression experiments could reflect the differences between *in vitro* and *in vivo* assays. Alternatively, the differences in results could be attributed to the divergent genetic backgrounds of the cell lines. The cell lines are all highly evolved cancer cell lines carrying numerous genetic and epigenetic aberrations (Mahlamäki et al., 2002; Sato et al., 2003a; Deer et al., 2010) that are likely to influence their behavior. A third possibility is that MED29 possesses a dual role in cancer, with both oncogenic and tumor-suppressive characteristics depending on the surrounding milieu. There are a few examples of other proteins with such a dualistic role in cancer, of which transforming growth factor beta (TGFβ) is perhaps the best known. TGFβ is frequently overexpressed in breast carcinomas, and its expression is associated with poor prognosis and metastasis (Ivanovic et al., 2003; Desruisseau et al., 2006; Ivanovic et al., 2006). TGFβ predominantly exerts tumor-suppressive properties that cancer cells must evade for malignant transformation; however, it can initiate immune evasion, angiogenesis and metastatic dissemination during tumor progression (Massague, 2008). Other examples of such dualistic proteins in cancer include the macrophage migration inhibitory factor (MIF) in breast cancer (Verjans et al., 2009) and the v-ski sarcoma viral oncogene homolog in pancreatic cancer (Wang et al., 2009). Also the Notch developmental signaling pathway has been implicated in several malignancies, acting in oncogenic or tumor suppressive fashion depending on the cellular context (reviewed by Yin et al., 2010).

Finally, genome-wide microarrays were utilized to explore MED29-induced differences in gene expression in MIA PaCa-2 and Hs 700T cells to clarify the underlying mechanisms and the role of MED29 in the regulation of growth and survival. Downregulation was more prevalent than upregulation among the differentially expressed genes, suggesting that MED29 functions mainly as a transcriptional repressor. Gene ontology and pathway analyses clearly indicated that the proteins encoded by the differentially expressed genes are involved in processes such as the cell cycle, cell division, cellular growth and proliferation, and cell death. Association with cell cycle regulation was most prominent, showing downregulation

of cell cycle promoting genes and upregulation of cell cycle regulatory genes. The microarray data were validated by a qRT-PCR-based method that confirmed the central role of MED29 in cell cycle regulation, with an overall inhibitory effect on cell cycle progression. This conclusion is in excellent concordance with our *in vivo* data demonstrating reduced tumor formation after MED29 induction.

4. The role of Mediator in cancer (III)

Gene transcription is a complex process regulating fundamental biological processes, such as growth and differentiation. It involves a myriad of specialized proteins and protein complexes that need to co-operate in a well-organized manner to express a given gene at a given time (Taatjes et al., 2004). Mediator acts in this process as a global transcriptional regulator mediating regulatory information from specific activators to the general transcription machinery; however, there is still lack of understanding regarding its function (Kornberg, 2005; Casamassimi & Napoli, 2007; Lewis, 2010). A large body of work has concentrated on determination of the structural composition of Mediator, which is now fairly well established (Casamassimi & Napoli., 2007). Further studies are needed to elucidate its mode of action. Recently, a multiple allosteric networks model (MANM) was applied to gain better insight into the function of Mediator (Lewis, 2010). According to this model, Mediator is suggested to exist not only as a physical but also as a functional network of interconnected proteins through which information is mediated from subunit to subunit by the propagation of a specific allosteric state. In addition, several distinct sub-networks within the Mediator complex defined by their intern connections were found to have discrete functions that were activated through interactions with different activator proteins (Lewis, 2010).

MED29 is a component of this large, multi-subunit complex, but its exact role remains elusive (Conaway et al., 2005b; Malik & Roeder, 2005). Based upon the roles of MED29 revealed by *in vivo* tumor formation, functional *in vitro* assays, and transcriptional regulation of several cell cycle- and cell division-related genes, it is reasonable to assume that it is involved in cancer pathogenesis. MED29 has been previously implicated as a transcriptional suppressor of SRE and AP-1, which are components of the MAPK signaling pathway, a cascade that is involved in multiple

cellular processes from growth to differentiation and apoptosis (Wang et al., 2004). The involvement of MED29 in the MAPK signaling cascade suggests a fundamental role for this protein in the regulation of growth and differentiation. Furthermore, the proposed suppressive role supports our findings from MED29 overexpression experiments, in which MED29 induction led to decreased cell growth and tumor formation as well as downregulation of several growth promoting genes.

MED29 has not been previously linked to any malignancies, but accumulating evidence indicates multiple subunits of Mediator in the development of various cancers. For example, CDK8 has been implicated as an oncogene and has a putative role in transcriptional regulation of key pathways in colon cancer (Firestein et al., 2008). In addition, CDK8 has been shown to have increased expression in colorectal tumors and a significant correlation with carcinogenesis, tumor progression and patient survival (Seo et al., 2010). Similarly, MED28 is upregulated in several cancers (Zhang et al., 2004; Yoon et al., 2010) and associated with a poor outcome in breast cancer (Yoon et al., 2010). Moreover, the inhibition of MED28 expression decreases cellular proliferation *in vitro* and *in vivo* (Lu et al., 2007). MED1 is amplified in breast and ovarian cancers (Zhu et al., 1999) and overexpressed in prostate cancer (Vijayvargia et al., 2007). Furthermore, the silencing of MED1 and MED17 inhibits the expression of androgen receptor target genes, decreases cellular proliferation, inhibits cell cycle progression, and increases apoptosis in prostate cancer (Vijayvargia et al., 2007). Moreover, Mediator has been reported to be a key transducer in several signaling pathways that are often affected in cancer, including Ras/MAP kinase (Stevens et al., 2002; Mo et al., 2004), Wnt/β-catenin (Kim et al., 2006), and TGFβ pathways (Kato et al., 2002). All of these observations substantiate the involvement of Mediator in cancer, which is not surprising in the context of transcriptional regulation.

5. Future prospects

This study was the first to characterize the 19q13 amplicon in pancreatic cancer in detail and to identify *MED29* as the most promising target gene. Using a large collection of primary tumors, a correlation between this amplicon and poor tumor phenotype in pancreatic cancer was also demonstrated for the first time. This

association is an important finding and the subsequent logical step would now be to evaluate MED29 protein levels in the same set of primary tumors. These analyses would reveal the direct clinical significance of MED29 in pancreatic cancer.

A dramatic phenotypic effect was observed in the subcutaneous mouse xenograft model using MED29-expressing pancreatic cancer cells. The next step could be the use of an orthotopic or genetically engineered mouse model to study the impact of MED29 manipulation in a more appropriate cellular environment. In addition, the *in vivo* effects of *MED29* silencing could be examined using pancreatic cancer cell lines with high endogenous expression. In such studies, both subcutaneous and orthotopic mouse models could be utilized in a similarly as in the overexpression analyses.

Considering the exciting novel findings in this study, it would be interesting to evaluate the role of down-stream effectors of MED29 in pancreatic cancer pathogenesis. The microarray data already pinpointed a range of interesting genes and pathways with MED29-regulated expression. Some of these have a known function in e.g. cell cycle regulation and thus are likely to have a relevant role in cancer, but others have not been previously linked to tumorigenesis and should thus be investigated in greater detail. Finally, given the central role of Mediator in transcriptional regulation, it would be appealing to study the possible role of other components of the Mediator in cancer pathogenesis.

CONCLUSIONS

The main aim of this study was to perform a detailed molecular characterization of the 19q13 amplification in pancreatic cancer, and more precisely, to elucidate the biological significance of this amplification and the role of individual genes that are activated through this amplification during tumor development.

Thorough amplicon mapping of the 19q13 locus revealed a minimal amplified region of 1.1 Mb and a highly amplified core region of 660 kb. The clinical significance of this amplicon core was evaluated in two sets of primary pancreatic tumors, both of which revealed 19q13 amplification to be a frequent event in pancreatic cancer, affecting more than 10% of patients. Importantly, the 19q13 copy number increases correlated with both tumor stage and grade. This is a new and important finding in pancreatic cancer. Patient survival was not significantly affected by this amplification, although the average survival time of patients with high-level amplification was notably shorter than the survival time of patients with normal copy number status.

Comprehensive expression analysis of putative amplification targets disclosed several overexpressed genes. However, the combined data from the expression survey and the RNAi-based viability screen for functional targets revealed *MED29*, a novel cancer-associated gene, to be the most promising target gene. It was consistently overexpressed, and its downregulation led to significant phenotypic changes in amplified pancreatic cancer cells. Further functional studies of MED29 revealed both oncogenic and tumor-suppressive characteristics. RNAi-based downregulation of high endogenous MED29 expression led to the inhibition of several tumor cell-associated phenotypes, including cell growth, migration, invasion, and colony formation, substantiating the oncogenic role of MED29 in pancreatic cancer. In contrast, the *in vivo* xenograft data from nude mice injected with pancreatic cancer cells with induced MED29 expression showed a dramatic reduction in tumor formation, thus providing a strong indication of a tumor suppressive role of MED29. Finally, systematic genome-wide expression analysis

indicated that these phenotypic effects are largely mediated by alterations in cell cycle regulation.

Taken together, this study provides new exciting information on molecular events in pancreatic cancer and serves as a valuable reminder of the contextual concept of cancer. The study accomplished detailed characterization of the 19q13 amplification in pancreatic cancer, including determination of its frequency and clinical significance, as well as the identification of a novel cancer-associated gene, *MED29*. It is fascinating that MED29 seems to possess a dual role in pancreatic cancer, having both oncogenic and tumor-suppressive properties. However, the further functional characterization of MED29 and its interacting partners are needed to elucidate its role in cancer more precisely. A deeper understanding of the Mediator complex and its individual components in transcriptional regulation might help to build a more comprehensive view of the functional role and mechanisms of action of MED29 in cancer pathogenesis.

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REFERENCES

Aguirre AJ, Bardeesy N, Sinha M, Lopez L, Tuveson DA, Horner J, Redston MS, DePinho RA. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. Genes Dev 2003;17:3112-3126.

Aguirre AJ, Brennan C, Bailey G, Sinha R, Feng B, Leo C, Zhang Y, Zhang J, Gans JD, Bardeesy N, Cauwels C, Cordon-Cardo C, Redston MS, DePinho RA, Chin L. High-resolution characterization of the pancreatic adenocarcinoma genome. Proc Natl Acad Sci U S A 2004;101:9067-9072.

Albertson DG. Gene amplification in cancer. Trends Genet 2006;22:447-455.

Albertson DG, Collins C, McCormick F, Gray JW. Chromosome aberrations in solid tumors. Nat Genet 2003;34:369-376.

Al-Kuraya K, Schraml P, Torhorst J, Tapia C, Zaharieva B, Novotny H, Spichtin H, Maurer R, Mirlacher M, Kochli O, Zuber M, Dieterich H, Mross F, Wilber K, Simon R, Sauter G. Prognostic relevance of gene amplifications and coamplifications in breast cancer. Cancer Res 2004;64:8534-8540.

Andersen CL, Hostetter G, Grigoryan A, Sauter G, Kallioniemi A. Improved procedure for fluorescence in situ hybridization on tissue microarrays. Cytometry 2001;45:83-86.

Andriole GL, Levin DL, Crawford ED, Gelmann EP, Pinsky PF, Chia D, Kramer BS, Reding D, Church TR, Grubb RL, Izmirlian G, Ragard LR, Clapp JD, Prorok PC, Gohagan JK, PLCO Project Team. Prostate Cancer Screening in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial: findings from the initial screening round of a randomized trial. J Natl Cancer Inst 2005;97:433-438.

Ansari SA, He Q, Morse RH. Mediator complex association with constitutively transcribed genes in yeast. Proc Natl Acad Sci U S A 2009;106:16734-16739.

Apple SK, Hecht JR, Lewin DN, Jahromi SA, Grody WW, Nieberg RK. Immunohistochemical evaluation of K-ras, p53, and HER-2/neu expression in hyperplastic, dysplastic, and carcinomatous lesions of the pancreas: evidence for multistep carcinogenesis. Hum Pathol 1999;30:123-129.

Atiye J, Wolf M, Kaur S, Monni O, Bohling T, Kivioja A, Tas E, Serra M, Tarkkanen M, Knuutila S. Gene amplifications in osteosarcoma-CGH microarray analysis. Genes Chromosomes Cancer 2005;42:158-163.

Balsara BR, Sonoda G, du Manoir S, Siegfried JM, Gabrielson E, Testa JR. Comparative genomic hybridization analysis detects frequent, often high-level,

overrepresentation of DNA sequences at 3q, 5p, 7p, and 8q in human non-small cell lung carcinomas. Cancer Res 1997;57:2116-2120.

Barbato C, Arisi I, Frizzo ME, Brandi R, Da Sacco L, Masotti A. Computational challenges in miRNA target predictions: to be or not to be a true target? J Biomed Biotechnol 2009:803069.

Bardeesy N, Cheng KH, Berger JH, Chu GC, Pahler J, Olson P, Hezel AF, Horner J, Lauwers GY, Hanahan D, DePinho RA. Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. Genes Dev. 2006;20:3130-46.

Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. Nat Rev Cancer 2002;2:897-909.

Bardi G, Johansson B, Pandis N, Mandahl N, Bak-Jensen E, Lindstrom C, Tornqvist A, Frederiksen H, Andren-Sandberg A, Mitelman F. Cytogenetic analysis of 52 colorectal carcinomas--non-random aberration pattern and correlation with pathologic parameters. Int J Cancer 1993;55:422-428.

Barlund M, Monni O, Kononen J, Cornelison R, Torhorst J, Sauter G, Kallioniemi O, Kallioniemi A. Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. Cancer Res 2000;60:5340-5344.

Barrett MT, Scheffer A, Ben-Dor A, Sampas N, Lipson D, Kincaid R, Tsang P, Curry B, Baird K, Meltzer PS, Yakhini Z, Bruhn L, Laderman S. Comparative genomic hybridization using oligonucleotide microarrays and total genomic DNA. Proc Natl Acad Sci U S A 2004;101:17765-17770.

Bartsch DK, Sina-Frey M, Lang S, Wild A, Gerdes B, Barth P, Kress R, Grutzmann R, Colombo-Benkmann M, Ziegler A, Hahn SA, Rothmund M, Rieder H. CDKN2A germline mutations in familial pancreatic cancer. Ann Surg 2002;236:730-737.

Bashyam MD, Bair R, Kim YH, Wang P, Hernandez-Boussard T, Karikari CA, Tibshirani R, Maitra A, Pollack JR. Array-based comparative genomic hybridization identifies localized DNA amplifications and homozygous deletions in pancreatic cancer. Neoplasia 2005;7:556-562.

Baumgart M, Heinmoller E, Horstmann O, Becker H, Ghadimi BM. The genetic basis of sporadic pancreatic cancer. Cell Oncol 2005;27:3-13.

Bell DW. Our changing view of the genomic landscape of cancer. J Pathol 2010;220:231-243.

Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J Cancer 1995;64:280-285.

Benjamini Y, Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc 1995;57:289–300.

Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, Parker AR, Shimada Y, Eshleman JR, Watkins DN, Beachy PA. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature 2003;425:846-851.

Bhaumik SR, Raha T, Aiello DP, Green MR. In vivo target of a transcriptional activator revealed by fluorescence resonance energy transfer. Genes Dev 2004;18:333-343.

Bissell MJ, Radisky D. Putting tumours in context. Nat Rev Cancer 2001;1:46-54.

Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C, Croce CM. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 2007;297:1901-1908.

Boube M, Joulia L, Cribbs DL, Bourbon HM. Evidence for a mediator of RNA polymerase II transcriptional regulation conserved from yeast to man. Cell 2002;110:143-151.

Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. PLoS Biol 2005;3:e85.

Brugge WR, Lauwers GY, Sahani D, Fernandez-del Castillo C,Warshaw AL. Cystic neoplasms of the pancreas. N Engl J Med 2004;351:1218-1226.

Bryant GO, Ptashne M. Independent recruitment in vivo by Gal4 of two complexes required for transcription. Mol Cell 2003;11:1301-1309.

Buchholz M, Gress TM. Molecular changes in pancreatic cancer. Expert Rev Anticancer Ther 2009;9:1487-1497.

Cai WW, Mao JH, Chow CW, Damani S, Balmain A, Bradley A. Genome-wide detection of chromosomal imbalances in tumors using BAC microarrays. Nat Biotechnol 2002;20:393-396.

Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857-866.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2002;99:15524-15529.

Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci U S A 1992;89:4285-4289.

Casamassimi A, Napoli C. Mediator complexes and eukaryotic transcription regulation: an overview. Biochimie 2007;89:1439-1446.

Chadick JZ, Asturias FJ. Structure of eukaryotic Mediator complexes. Trends Biochem Sci 2005;30:264-271.

Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001;410:37-40.

Chase BA, Baker BS. A genetic analysis of intersex, a gene regulating sexual differentiation in Drosophila melanogaster females. Genetics 1995;139:1649-1661.

Chen S, Auletta T, Dovirak O, Hutter C, Kuntz K, El-Ftesi S, Kendall J, Han H, Von Hoff DD, Ashfaq R, Maitra A, Iacobuzio-Donahue CA, Hruban RH, Lucito R. Copy number alterations in pancreatic cancer identify recurrent PAK4 amplification. Cancer Biol Ther 2008;7:

Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, Testa JR. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci U S A 1992;89:9267-9271.

Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK, Testa JR. Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Proc Natl Acad Sci U S A 1996;93:3636-3641.

Chu GC, Kimmelman AC, Hezel AF, DePinho RA. Stromal biology of pancreatic cancer. J Cell Biochem 2007;101:887-907.

Conaway JW, Florens L, Sato S, Tomomori-Sato C, Parmely TJ, Yao T, Swanson SK, Banks CA, Washburn MP, Conaway RC. The mammalian Mediator complex. FEBS Lett 2005a;579:904-908.

Conaway RC, Sato S, Tomomori-Sato C, Yao T, Conaway JW. The mammalian Mediator complex and its role in transcriptional regulation. Trends Biochem Sci 2005b;30:250-255.

Cook WD, McCaw BJ. Accommodating haploinsufficient tumor suppressor genes in Knudson's model. Oncogene 2000;19:3434-3438.

Coquelle A, Pipiras E, Toledo F, Buttin G, Debatisse M. Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplicons. Cell 1997;89:215-225.

Croce CM. Oncogenes and cancer. N Engl J Med 2008;358:502-511.

Day JD, Digiuseppe JA, Yeo C, Lai-Goldman M, Anderson SM, Goodman SN, Kern SE, Hruban RH. Immunohistochemical evaluation of HER-2/neu expression in pancreatic adenocarcinoma and pancreatic intraepithelial neoplasms. Hum Pathol 1996;27:119-124.

Deer EL, Gonzalez-Hernandez J, Coursen JD, Shea JE, Ngatia J, Scaife CL, Firpo MA, Mulvihill SJ. Phenotype and genotype of pancreatic cancer cell lines.

Pancreas 2010;39:425-435.

Deng X, Ewton DZ, Li S, Naqvi A, Mercer SE, Landas S, Friedman E. The kinase Mirk/Dyrk1B mediates cell survival in pancreatic ductal adenocarcinoma. Cancer Res 2006;66:4149-4158.

Dennis G,Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC,Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 2003;4:P3.

Desruisseau S, Palmari J, Giusti C, Romain S, Martin PM, Berthois Y. Determination of TGFbeta1 protein level in human primary breast cancers and its relationship with survival. Br J Cancer 2006;94:239-246.

Dohna M, Reincke M, Mincheva A, Allolio B, Solinas-Toldo S, Lichter P. Adrenocortical carcinoma is characterized by a high frequency of chromosomal gains and high-level amplifications. Genes Chromosomes Cancer 2000;28:145-152.

Dotson MR, Yuan CX, Roeder RG, Myers LC, Gustafsson CM, Jiang YW, Li Y, Kornberg RD, Asturias FJ. Structural organization of yeast and mammalian mediator complexes. Proc Natl Acad Sci U S A 2000;97:14307-14310.

Eberle MA, Pfutzer R, Pogue-Geile KL, Bronner MP, Crispin D, Kimmey MB, Duerr RH, Kruglyak L, Whitcomb DC, Brentnall TA. A new susceptibility locus for autosomal dominant pancreatic cancer maps to chromosome 4q32-34. Am J Hum Genet 2002;70:1044-1048.

Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 2001;15:188-200.

Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259-269.

Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007;8:286-298.

Everhart J, Wright D. Diabetes mellitus as a risk factor for pancreatic cancer. A meta-analysis. JAMA 1995;273:1605-1609.

Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. Nat Rev Genet 2006;7:21-33.

Fero ML, Randel E, Gurley KE, Roberts JM, Kemp CJ. The murine gene p27Kip1 is haplo-insufficient for tumour suppression. Nature 1998;396:177-180.

Feuk L, Carson AR, Scherer SW. Structural variation in the human genome. Nat Rev Genet 2006;7:85-97.

Firestein R, Bass AJ, Kim SY, Dunn IF, Silver SJ, Guney I, Freed E, Ligon AH, Vena N, Ogino S, Chheda MG, Tamayo P, Finn S, Shrestha Y, Boehm JS, Jain S, Bojarski E, Mermel C, Barretina J, Chan JA, Baselga J, Tabernero J, Root DE,

Fuchs CS, Loda M, Shivdasani RA, Meyerson M, Hahn WC. CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity. Nature 2008;455:547-551.

Flanagan PM, Kelleher RJ,3rd, Sayre MH, Tschochner H, Kornberg RD. A mediator required for activation of RNA polymerase II transcription in vitro. Nature 1991;350:436-438.

Freitas D, Fernandes Gdos S, Hoff PM, Cunha JE. Medical management of pancreatic adenocarcinoma. Pancreatology 2009;9:223-232.

Friess H, Yamanaka Y, Kobrin MS, Do DA, Buchler MW, Korc M. Enhanced erbB-3 expression in human pancreatic cancer correlates with tumor progression. Clin Cancer Res 1995;1:1413-1420.

Fuchs CS, Colditz GA, Stampfer MJ, Giovannucci EL, Hunter DJ, Rimm EB, Willett WC, Speizer FE. A prospective study of cigarette smoking and the risk of pancreatic cancer. Arch Intern Med 1996;156:2255-2260.

Ganguly A, Nichols KE, Grant G, Rappaport E, Shields C. Molecular karyotype of sporadic unilateral retinoblastoma tumors. Retina 2009;29:1002-1012.

Gansauge S, Gansauge F, Ramadani M, Stobbe H, Rau B, Harada N, Beger HG. Overexpression of cyclin D1 in human pancreatic carcinoma is associated with poor prognosis. Cancer Res 1997;57:1634-1637.

Garrett-Engele CM, Siegal ML, Manoli DS, Williams BC, Li H, Baker BS. intersex, a gene required for female sexual development in Drosophila, is expressed in both sexes and functions together with doublesex to regulate terminal differentiation. Development 2002;129:4661-4675.

Geary J, Sasieni P, Houlston R, Izatt L, Eeles R, Payne SJ, Fisher S, Hodgson SV. Gene-related cancer spectrum in families with hereditary non-polyposis colorectal cancer (HNPCC). Fam Cancer 2008;7:163-172.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004;5:R80.

Giardiello FM, Brensinger JD, Tersmette AC, Goodman SN, Petersen GM, Booker SV, Cruz-Correa M, Offerhaus JA. Very high risk of cancer in familial Peutz-Jeghers syndrome. Gastroenterology 2000;119:1447-1453.

Giardiello FM, Offerhaus GJ, Lee DH, Krush AJ, Tersmette AC, Booker SV, Kelley NC, Hamilton SR. Increased risk of thyroid and pancreatic carcinoma in familial adenomatous polyposis. Gut 1993;34:1394-1396.

Gisselsson D, Pettersson L, Hoglund M, Heidenblad M, Gorunova L, Wiegant J, Mertens F, Dal Cin P, Mitelman F, Mandahl N. Chromosomal breakage-fusion-bridge events cause genetic intratumor heterogeneity. Proc Natl Acad Sci U S A

2000;97:5357-5362.

Goggins M, Schutte M, Lu J, Moskaluk CA, Weinstein CL, Petersen GM, Yeo CJ, Jackson CE, Lynch HT, Hruban RH, Kern SE. Germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas. Cancer Res 1996;56:5360-5364.

Goldstein AM, Struewing JP, Fraser MC, Smith MW,Tucker MA. Prospective risk of cancer in CDKN2A germline mutation carriers. J Med Genet 2004;41:421-424.

Gorunova L, Hoglund M, Andren-Sandberg A, Dawiskiba S, Jin Y, Mitelman F, Johansson B. Cytogenetic analysis of pancreatic carcinomas: intratumor heterogeneity and nonrandom pattern of chromosome aberrations. Genes Chromosomes Cancer 1998;23:81-99.

Govind CK, Yoon S, Qiu H, Govind S, Hinnebusch AG. Simultaneous recruitment of coactivators by Gcn4p stimulates multiple steps of transcription in vivo. Mol Cell Biol 2005;25:5626-5638.

Griffin CA, Hruban RH, Morsberger LA, Ellingham T, Long PP, Jaffee EM, Hauda KM, Bohlander SK, Yeo CJ. Consistent chromosome abnormalities in adenocarcinoma of the pancreas. Cancer Res 1995;55:2394-2399.

Gysin S, Rickert P, Kastury K, McMahon M. Analysis of genomic DNA alterations and mRNA expression patterns in a panel of human pancreatic cancer cell lines. Genes Chromosomes Cancer 2005;44:37-51.

Hahn PJ. Molecular biology of double-minute chromosomes. Bioessays 1993;15:477-484.

Hahn S. Structure and mechanism of the RNA polymerase II transcription machinery. Nat Struct Mol Biol 2004;11:394-403.

Hahn S, Roberts S. The zinc ribbon domains of the general transcription factors TFIIB and Brf: conserved functional surfaces but different roles in transcription initiation. Genes Dev 2000;14:719-730.

Han SJ, Lee YC, Gim BS, Ryu GH, Park SJ, Lane WS, Kim YJ. Activator-specific requirement of yeast mediator proteins for RNA polymerase II transcriptional activation. Mol Cell Biol 1999;19:979-988.

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.

Hansel DE, Kern SE, Hruban RH. Molecular pathogenesis of pancreatic cancer. Annu Rev Genomics Hum Genet 2003;4:237-256.

Harada T, Chelala C, Bhakta V, Chaplin T, Caulee K, Baril P, Young BD, Lemoine NR. Genome-wide DNA copy number analysis in pancreatic cancer using high-density single nucleotide polymorphism arrays. Oncogene 2008;27:1951-1960.

Harada T, Okita K, Shiraishi K, Kusano N, Kondoh S, Sasaki K. Interglandular

cytogenetic heterogeneity detected by comparative genomic hybridization in pancreatic cancer. Cancer Res 2002;62:835-839.

Harsha HC, Kandasamy K, Ranganathan P, Rani S, Ramabadran S, Gollapudi S, Balakrishnan L, Dwivedi SB, Telikicherla D, Selvan LD, Goel R, Mathivanan S, Marimuthu A, Kashyap M, Vizza RF, Mayer RJ, Decaprio JA, Srivastava S, Hanash SM, Hruban RH, Pandey A. A compendium of potential biomarkers of pancreatic cancer. PLoS Med 2009;6:e1000046.

Heidenblad M, Lindgren D, Veltman JA, Jonson T, Mahlamaki EH, Gorunova L, van Kessel AG, Schoenmakers EF, Hoglund M. Microarray analyses reveal strong influence of DNA copy number alterations on the transcriptional patterns in pancreatic cancer: implications for the interpretation of genomic amplifications. Oncogene 2005;24:1794-1801.

Heidenblad M, Schoenmakers EF, Jonson T, Gorunova L, Veltman JA, van Kessel AG, Hoglund M. Genome-wide array-based comparative genomic hybridization reveals multiple amplification targets and novel homozygous deletions in pancreatic carcinoma cell lines. Cancer Res 2004;64:3052-3059.

Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 2007;1:313-323.

Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, DePinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. Genes Dev 2006;20:1218-1249.

Hidalgo M. Pancreatic cancer. N Engl J Med 2010;362:1605-1617.

Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, Kawaguchi Y, Johann D, Liotta LA, Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 2003;4:437-450.

Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, Rustgi AK, Chang S, Tuveson DA. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell 2005;7:469-483.

Hodgson JG, Chin K, Collins C, Gray JW. Genome amplification of chromosome 20 in breast cancer. Breast Cancer Res Treat 2003;78:337-345.

Hoglund M, Gorunova L, Andren-Sandberg A, Dawiskiba S, Mitelman F, Johansson B. Cytogenetic and fluorescence in situ hybridization analyses of chromosome 19 aberrations in pancreatic carcinomas: frequent loss of 19p13.3 and gain of 19q13.1-13.2. Genes Chromosomes Cancer 1998;21:8-16.

Holzmann K, Kohlhammer H, Schwaenen C, Wessendorf S, Kestler HA, Schwoerer A, Rau B, Radlwimmer B, Dohner H, Lichter P, Gress T, Bentz M. Genomic

DNA-chip hybridization reveals a higher incidence of genomic amplifications in pancreatic cancer than conventional comparative genomic hybridization and leads to the identification of novel candidate genes. Cancer Res 2004;64:4428-4433.

Hruban RH, Goggins M, Parsons J, Kern SE. Progression model for pancreatic cancer. Clin Cancer Res 2000;6:2969-2972.

Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nat Protoc 2009;4:44–57.

Huntsman DG, Chin SF, Muleris M, Batley SJ, Collins VP, Wiedemann LM, Aparicio S, Caldas C. MLL2, the second human homolog of the Drosophila trithorax gene, maps to 19q13.1 and is amplified in solid tumor cell lines. Oncogene 1999;18:7975-7984.

Huxley R, Ansary-Moghaddam A, Berrington de Gonzalez A, Barzi F, Woodward M. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. Br J Cancer 2005;92:2076-2083.

Hyman E, Kauraniemi P, Hautaniemi S, Wolf M, Mousses S, Rozenblum E, Ringner M, Sauter G, Monni O, Elkahloun A, Kallioniemi OP, Kallioniemi A. Impact of DNA amplification on gene expression patterns in breast cancer. Cancer Res 2002;62:6240-6245.

Ivanovic V, Demajo M, Krtolica K, Krajnovic M, Konstantinovic M, Baltic V, Prtenjak G, Stojiljkovic B, Breberina M, Neskovic-Konstantinovic Z, Nikolic-Vukosavljevic D, Dimitrijevic B. Elevated plasma TGF-beta1 levels correlate with decreased survival of metastatic breast cancer patients. Clin Chim Acta 2006;371:191-193.

Ivanovic V, Todorovic-Rakovic N, Demajo M, Neskovic-Konstantinovic Z, Subota V, Ivanisevic-Milovanovic O, Nikolic-Vukosavljevic D. Elevated plasma levels of transforming growth factor-beta 1 (TGF-beta 1) in patients with advanced breast cancer: association with disease progression. Eur J Cancer 2003;39:454-461.

Janik P, Briand P, Hartmann NR. The effect of estrone-progesterone treatment on cell proliferation kinetics of hormone-dependent GR mouse mammary tumors. Cancer Res 1975;35:3698-3704.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225-249.

Jiao L, Mitrou PN, Reedy J, Graubard BI, Hollenbeck AR, Schatzkin A, Stolzenberg-Solomon R. A combined healthy lifestyle score and risk of pancreatic cancer in a large cohort study. Arch Intern Med 2009;169:764-770.

Johansson B, Bardi G, Heim S, Mandahl N, Mertens F, Bak-Jensen E, Andren-Sandberg A, Mitelman F. Nonrandom chromosomal rearrangements in pancreatic carcinomas. Cancer 1992;69:1674-1681.

Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683-692.

Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 2008;321:1801-1806.

Kallioniemi A, Kallioniemi OP, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW, Waldman FM. Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. Proc Natl Acad Sci U S A 1994;91:2156-2160.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 1992;258:818-821.

Kato Y, Habas R, Katsuyama Y, Naar AM,He X. A component of the ARC/Mediator complex required for TGF beta/Nodal signalling. Nature 2002;418:641-646.

Kauraniemi P, Barlund M, Monni O, Kallioniemi A. New amplified and highly expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA microarrays. Cancer Res 2001;61:8235-8240.

Kikuchi S, Honda K, Tsuda H, Hiraoka N, Imoto I, Kosuge T, Umaki T, Onozato K, Shitashige M, Yamaguchi U, Ono M, Tsuchida A, Aoki T, Inazawa J, Hirohashi S, Yamada T. Expression and gene amplification of actinin-4 in invasive ductal carcinoma of the pancreas. Clin Cancer Res 2008;14:5348-5356.

Kim S, Xu X, Hecht A, Boyer TG. Mediator is a transducer of Wnt/beta-catenin signaling. J Biol Chem 2006;281:14066-14075.

Kim TM, Yim SH, Lee JS, Kwon MS, Ryu JW, Kang HM, Fiegler H, Carter NP, Chung YJ. Genome-wide screening of genomic alterations and their clinicopathologic implications in non-small cell lung cancers. Clin Cancer Res 2005;11:8235-8242.

Kim YJ, Bjorklund S, Li Y, Sayre MH, Kornberg RD. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell 1994;77:599-608.

Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159-170.

Kinzler KW, Vogelstein B. Cancer-susceptibility genes. Gatekeepers and caretakers. Nature 1997;386:761, 763.

Kinzler KW, Vogelstein B. Landscaping the cancer terrain. Science 1998;280:1036-

1037.

Kiviluoto. Voiko haiman duktaalisesta adenokarsinoomasta parantua? Duodecim 2008,124:2410-2411.

Kleihues P, Schauble B, zur Hausen A, Esteve J, Ohgaki H. Tumors associated with p53 germline mutations: a synopsis of 91 families. Am J Pathol 1997;150:1-13.

Klein AP, Brune KA, Petersen GM, Goggins M, Tersmette AC, Offerhaus GJ, Griffin C, Cameron JL, Yeo CJ, Kern S, Hruban RH. Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. Cancer Res 2004;64:2634-2638.

Klöppel, G, Solcia, E, Longnecker, DS, Capella C, Sobin LH. Histologic Typing of Tumours of the Exocrine Pancreas. Springer-Verlag, New York 1996.

Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A. 1971;68:820-823.

Knösel T, Schlüns K, Stein U, Schwabe H, Schlag PM, Dietel M, Petersen I. Chromosomal alterations during lymphatic and liver metastasis formation of colorectal cancer. Neoplasia. 2004;6:23-28

Koch F, Jourquin F, Ferrier P, Andrau JC. Genome-wide RNA polymerase II: not genes only! Trends Biochem Sci 2008;33:265-273.

Kokubo T, Swanson MJ, Nishikawa JI, Hinnebusch AG, Nakatani Y. The yeast TAF145 inhibitory domain and TFIIA competitively bind to TATA-binding protein. Mol Cell Biol 1998;18:1003-1012.

Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844-847.

Kornberg RD. Mediator and the mechanism of transcriptional activation. Trends Biochem Sci 2005;30:235-239.

Kouzarides T. Chromatin modifications and their function. Cell 2007;128:693-705.

Kuras L, Borggrefe T, Kornberg RD. Association of the Mediator complex with enhancers of active genes. Proc Natl Acad Sci U S A 2003;100:13887-13891.

Larramendy ML, Tarkkanen M, Valle J, Kivioja AH, Ervasti H, Karaharju E, Salmivalli T, Elomaa I, Knuutila S. Gains, losses, and amplifications of DNA sequences evaluated by comparative genomic hybridization in chondrosarcomas. Am J Pathol 1997;150:685-691.

Larsson SC, Orsini N, Wolk A. Body mass index and pancreatic cancer risk: A meta-analysis of prospective studies. Int J Cancer 2007;120:1993-1998.

Laurila E, Savinainen K, Kuuselo R, Karhu R, Kallioniemi A. Characterization of the 7q21-q22 amplicon identifies ARPC1A, a subunit of the Arp2/3 complex, as

a regulator of cell migration and invasion in pancreatic cancer. Genes Chromosomes Cancer 2009;48:330-339.

Lee CJ, Dosch J, Simeone DM. Pancreatic cancer stem cells. J Clin Oncol 2008;26:2806-2812.

Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, Morgan DL, Postier RG, Brackett DJ, Schmittgen TD. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer 2007;120:1046-1054.

Levin B, Lieberman DA, McFarland B, Andrews KS, Brooks D, Bond J, Dash C, Giardiello FM, Glick S, Johnson D, Johnson CD, Levin TR, Pickhardt PJ, Rex DK, Smith RA, Thorson A, Winawer SJ, American Cancer Society Colorectal Cancer Advisory Group, US Multi-Society Task Force, American College of Radiology Colon Cancer Committee. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. Gastroenterology 2008;134:1570-1595.

Lewis BA. Understanding large multiprotein complexes: applying a multiple allosteric networks model to explain the function of the Mediator transcription complex. J Cell Sci 2010;123:159-163.

Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15-20.

Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. Cancer Res 2007;67:1030-1037.

Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 2005;433:769-773.

Lochan R, Daly AK, Reeves HL, Charnley RM. Genetic susceptibility in pancreatic ductal adenocarcinoma. Br J Surg 2008;95:22-32.

Lowenfels AB, Maisonneuve P. Risk factors for pancreatic cancer. J Cell Biochem 2005;95:649-656.

Lowenfels AB, Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, Dimagno EP, Andren-Sandberg A, Domellof L. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. N Engl J Med 1993;328:1433-1437.

Lowenfels AB, Maisonneuve P, DiMagno EP, Elitsur Y, Gates LK, Perrault J, Whitcomb DC. Hereditary pancreatitis and the risk of pancreatic cancer. International Hereditary Pancreatitis Study Group. J Natl Cancer Inst 1997;89:442-446.

Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature 2005;435:834-838.

Lu M, Sartippour MR, Zhang L, Norris AJ, Brooks MN. Targeted inhibition of EG-1 blocks breast tumor growth. Cancer Biol Ther 2007;6:936-941.

Lynch HT, Deters CA, Snyder CL, Lynch JF, Villeneuve P, Silberstein J, Martin H, Narod SA, Brand RE. BRCA1 and pancreatic cancer: pedigree findings and their causal relationships. Cancer Genet Cytogenet 2005;158:119-125.

Lynch HT, Smyrk T, Kern SE, Hruban RH, Lightdale CJ, Lemon SJ, Lynch JF, Fusaro LR, Fusaro RM, Ghadirian P. Familial pancreatic cancer: a review. Semin Oncol 1996;23:251-275.

Macleod K. Tumor suppressor genes. Curr Opin Genet Dev 2000;10:81-93.

Mahadevan D, Von Hoff DD. Tumor-stroma interactions in pancreatic ductal adenocarcinoma. Mol Cancer Ther 2007;6:1186-1197.

Mahlamaki EH, Barlund M, Tanner M, Gorunova L, Hoglund M, Karhu R, Kallioniemi A. Frequent amplification of 8q24, 11q, 17q, and 20q-specific genes in pancreatic cancer. Genes Chromosomes Cancer 2002;35:353-358.

Mahlamaki EH, Kauraniemi P, Monni O, Wolf M, Hautaniemi S, Kallioniemi A. High-resolution genomic and expression profiling reveals 105 putative amplification target genes in pancreatic cancer. Neoplasia 2004;6:432-439.

Maisonneuve P, Marshall BC, Lowenfels AB. Risk of pancreatic cancer in patients with cystic fibrosis. Gut 2007;56:1327-1328.

Maitra A, Fukushima N, Takaori K, Hruban RH. Precursors to invasive pancreatic cancer. Adv Anat Pathol 2005;12:81-91.

Maitra A, Hruban RH. Pancreatic cancer. Annu Rev Pathol 2008;3:157-188.

Maitra A, Kern SE, Hruban RH. Molecular pathogenesis of pancreatic cancer. Best Pract Res Clin Gastroenterol 2006;20:211-226.

Malik S, Roeder RG. Dynamic regulation of pol II transcription by the mammalian Mediator complex. Trends Biochem Sci 2005;30:256-263.

Malik S, Roeder RG. Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. Trends Biochem Sci 2000;25:277-283.

Malumbres M, Barbacid M. RAS oncogenes: the first 30 years. Nat Rev Cancer 2003;3:459-465.

Marchio A, Meddeb M, Pineau P, Danglot G, Tiollais P, Bernheim A, Dejean A. Recurrent chromosomal abnormalities in hepatocellular carcinoma detected by comparative genomic hybridization. Genes Chromosomes Cancer 1997;18:59-65.

Masamune A, Shimosegawa T. Signal transduction in pancreatic stellate cells. J Gastroenterol 2009;44:249-260.

Masood S, Bui MM. Prognostic and predictive value of HER2/neu oncogene in breast cancer. Microsc Res Tech 2002;59:102-108.

Mass RD, Press MF, Anderson S, Cobleigh MA, Vogel CL, Dybdal N, Leiberman G, Slamon DJ. Evaluation of clinical outcomes according to HER2 detection by fluorescence in situ hybridization in women with metastatic breast cancer treated with trastuzumab. Clin Breast Cancer 2005;6:240-246.

Massague J. TGFbeta in Cancer. Cell 2008;134:215-230.

McClintock B. The Fusion of Broken Ends of Chromosomes Following Nuclear Fusion. Proc Natl Acad Sci U S A 1942;28:458-463.

Medina PP, Castillo SD, Blanco S, Sanz-Garcia M, Largo C, Alvarez S, Yokota J, Gonzalez-Neira A, Benitez J, Clevers HC, Cigudosa JC, Lazo PA, Sanchez-Cespedes M. The SRY-HMG box gene, SOX4, is a target of gene amplification at chromosome 6p in lung cancer. Hum Mol Genet 2009;18:1343-1352.

Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature 2004;431:343-349.

Michaud DS, Giovannucci E, Willett WC, Colditz GA, Stampfer MJ, Fuchs CS. Physical activity, obesity, height, and the risk of pancreatic cancer. JAMA 2001;286:921-929.

Mihaljevic AL, Michalski CW, Friess H, Kleeff J. Molecular mechanism of pancreatic cancer--understanding proliferation, invasion, and metastasis. Langenbecks Arch Surg 2010;395:295-308.

Miwa W, Yasuda J, Murakami Y, Yashima K, Sugano K, Sekine T, Kono A, Egawa S, Yamaguchi K, Hayashizaki Y, Sekiya T. Isolation of DNA sequences amplified at chromosome 19q13.1-q13.2 including the AKT2 locus in human pancreatic cancer. Biochem Biophys Res Commun 1996;225:968-974.

Miyamoto Y, Maitra A, Ghosh B, Zechner U, Argani P, Iacobuzio-Donahue CA, Sriuranpong V, Iso T, Meszoely IM, Wolfe MS, Hruban RH, Ball DW, Schmid RM, Leach SD. Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. Cancer Cell 2003;3:565-576.

Mo X, Kowenz-Leutz E, Xu H, Leutz A. Ras induces mediator complex exchange on C/EBP beta. Mol Cell 2004;13:241-250.

Moniaux N, Nemos C, Schmied BM, Chauhan SC, Deb S, Morikane K, Choudhury A, Vanlith M, Sutherlin M, Sikela JM, Hollingsworth MA, Batra SK. The human homologue of the RNA polymerase II-associated factor 1 (hPaf1), localized on the 19q13 amplicon, is associated with tumorigenesis. Oncogene 2006;25:3247-3257.

Morris JP 4th, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. Nat Rev Cancer 2010;10:683-695.

Murnane JP, Sabatier L. Chromosome rearrangements resulting from telomere dysfunction and their role in cancer. Bioessays 2004;26:1164-1174.

Myers LC, Kornberg RD. Mediator of transcriptional regulation. Annu Rev Biochem 2000;69:729-749.

Myllykangas S, Knuutila S. Manifestation, mechanisms and mysteries of gene amplifications. Cancer Lett 2006;232:79-89.

Nambiar M, Kari V, Raghavan SC. Chromosomal translocations in cancer. Biochim Biophys Acta 2008;1786:139-152.

Narayan G, Bourdon V, Chaganti S, Arias-Pulido H, Nandula SV, Rao PH, Gissmann L, Durst M, Schneider A, Pothuri B, Mansukhani M, Basso K, Chaganti RS, Murty VV. Gene dosage alterations revealed by cDNA microarray analysis in cervical cancer: identification of candidate amplified and overexpressed genes. Genes Chromosomes Cancer 2007;46:373-384.

Ngo VN, Davis RE, Lamy L, Yu X, Zhao H, Lenz G, Lam LT, Dave S, Yang L, Powell J, Staudt LM. A loss-of-function RNA interference screen for molecular targets in cancer. Nature 2006;441:106-110.

Nowak NJ, Gaile D, Conroy JM, McQuaid D, Cowell J, Carter R, Goggins MG, Hruban RH, Maitra A. Genome-wide aberrations in pancreatic adenocarcinoma. Cancer Genet Cytogenet 2005;161:36-50.

Ohkuma Y. Multiple functions of general transcription factors TFIIE and TFIIH in transcription: possible points of regulation by trans-acting factors. J Biochem 1997;122:481-489.

Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999;99:247-257.

Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 1998;19:219-220.

Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, Madhu B, Goldgraben MA, Caldwell ME, Allard D, Frese KK, Denicola G, Feig C, Combs C, Winter SP, Ireland-Zecchini H, Reichelt S, Howat WJ, Chang A, Dhara M, Wang L, Rückert F, Grützmann R, Pilarsky C, Izeradjene K, Hingorani SR, Huang P, Davies SE, Plunkett W, Egorin M, Hruban RH, Whitebread N, McGovern K, Adams J, Iacobuzio-Donahue C, Griffiths J, Tuveson DA. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science. 2009;324:1457-1461.

Omura N, Goggins M. Epigenetics and epigenetic alterations in pancreatic cancer. Int J Clin Exp Pathol 2009;2:310-326.

Pasca di Magliano M, Biankin AV, Heiser PW, Cano DA, Gutierrez PJ, Deramaudt T, Segara D, Dawson AC, Kench JG, Henshall SM, Sutherland RL, Dlugosz A, Rustgi AK, Hebrok M. Common activation of canonical Wnt signaling in pancreatic adenocarcinoma. PLoS One 2007;2:e1155.

Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.

Parsa I, Longnecker DS, Scarpelli DG, Pour P, Reddy JK, Lefkowitz M. Ductal metaplasia of human exocrine pancreas and its association with carcinoma. Cancer Res 1985;45:1285-1290.

Pärssinen J, Kuukasjarvi T, Karhu R, Kallioniemi A. High-level amplification at 17q23 leads to coordinated overexpression of multiple adjacent genes in breast cancer. Br J Cancer 2007;96:1258-1264.

Petersen I, Langreck H, Wolf G, Schwendel A, Psille R, Vogt P, Reichel MB, Ried T, Dietel M. Small-cell lung cancer is characterized by a high incidence of deletions on chromosomes 3p, 4q, 5q, 10q, 13q and 17p. Br J Cancer 1997;75:79-86.

Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, Brown PO. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genet 1999;23:41-46.

Polyak K, Haviv I, Campbell IG. Co-evolution of tumor cells and their microenvironment. Trends Genet 2009;25:30-38.

Ponder BA. Cancer genetics. Nature 2001;411:336-341.

Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL, Visakorpi T. MicroRNA expression profiling in prostate cancer. Cancer Res 2007;67:6130-6135.

Raimondi S, Maisonneuve P, Lowenfels AB. Epidemiology of pancreatic cancer: an overview. Nat Rev Gastroenterol Hepatol 2009;6:699-708.

Ranganathan P, Harsha HC, Pandey A. Molecular alterations in exocrine neoplasms of the pancreas. Arch Pathol Lab Med 2009;133:405-412.

Rao PH, Arias-Pulido H, Lu XY, Harris CP, Vargas H, Zhang FF, Narayan G, Schneider A, Terry MB, Murty VV. Chromosomal amplifications, 3q gain and deletions of 2q33-q37 are the frequent genetic changes in cervical carcinoma. BMC Cancer 2004;4:5.

Reya T, Morrison SJ, Clarke MF,Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105-111.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nat Biotechnol 2004;22:326-330.

Richter J, Wagner U, Kononen J, Fijan A, Bruderer J, Schmid U, Ackermann D,

Maurer R, Alund G, Knonagel H, Rist M, Wilber K, Anabitarte M, Hering F, Hardmeier T, Schonenberger A, Flury R, Jager P, Fehr JL, Schraml P, Moch H, Mihatsch MJ, Gasser T, Kallioniemi OP, Sauter G. High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. Am J Pathol 2000;157:787-794.

Ried T, Petersen I, Holtgreve-Grez H, Speicher MR, Schrock E, du Manoir S, Cremer T. Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. Cancer Res 1994;54:1801-1806.

Ritchie ME, Silver J, Oshlack A, Holmes M, Diyagama D, Holloway A, Smyth GK. A comparison of background correction methods for two-colour microarrays. Bioinformatics 2007;23:2700-2707.

Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL, Lee DC. Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell 1990;61:1121-1135.

Santarius T, Shipley J, Brewer D, Stratton MR, Cooper CS. A census of amplified and overexpressed human cancer genes. Nat Rev Cancer 2010;10:59-64.

Saslow D, Boetes C, Burke W, Harms S, Leach MO, Lehman CD, Morris E, Pisano E, Schnall M, Sener S, Smith RA, Warner E, Yaffe M, Andrews KS, Russell CA, American Cancer Society Breast Cancer Advisory Group. American Cancer Society guidelines for breast screening with MRI as an adjunct to mammography. CA Cancer J Clin 2007;57:75-89.

Sato N, Fukushima N, Maitra A, Matsubayashi H, Yeo CJ, Cameron JL, Hruban RH, Goggins M. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. Cancer Res 2003;63:3735-3742.

Sato S, Tomomori-Sato C, Banks CA, Parmely TJ, Sorokina I, Brower CS, Conaway RC, Conaway JW. A mammalian homolog of Drosophila melanogaster transcriptional coactivator intersex is a subunit of the mammalian Mediator complex. J Biol Chem 2003;278:49671-49674.

Sato S, Tomomori-Sato C, Parmely TJ, Florens L, Zybailov B, Swanson SK, Banks CA, Jin J, Cai Y, Washburn MP, Conaway JW, Conaway RC. A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. Mol Cell 2004;14:685-691.

Savelyeva L, Schwab M. Amplification of oncogenes revisited: from expression profiling to clinical application. Cancer Lett 2001;167:115-123.

Schenk M, Schwartz AG, O'Neal E, Kinnard M, Greenson JK, Fryzek JP, Ying GS, Garabrant DH. Familial risk of pancreatic cancer. J Natl Cancer Inst. 2001;93:640-644.

Schleger C, Arens N, Zentgraf H, Bleyl U, Verbeke C. Identification of frequent chromosomal aberrations in ductal adenocarcinoma of the pancreas by

comparative genomic hybridization (CGH). J Pathol 2000;191:27-32.

Schleger C, Verbeke C, Hildenbrand R, Zentgraf H, Bleyl U. c-MYC activation in primary and metastatic ductal adenocarcinoma of the pancreas: incidence, mechanisms, and clinical significance. Mod Pathol 2002;15:462-469.

Schnelldorfer T, Ware AL, Sarr MG, Smyrk TC, Zhang L, Qin R, Gullerud RE, Donohue JH, Nagorney DM, Farnell MB. Long-term survival after pancreatoduodenectomy for pancreatic adenocarcinoma: is cure possible? Ann Surg 2008;24:456-462.

Schuller HM. Mechanisms of smoking-related lung and pancreatic adenocarcinoma development. Nat Rev Cancer 2002;2:455-463.

Schwab M. Oncogene amplification in solid tumors. Semin Cancer Biol 1999;9:319-325.

Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 1985;313:1111-1116.

Seo JO, Han SI, Lim SC. Role of CDK8 and beta-catenin in colorectal adenocarcinoma. Oncol Rep 2010;24:285-291.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593-602.

Sherr CJ. Principles of tumor suppression. Cell 2004;116:235-246.

Shimizu N, Shingaki K, Kaneko-Sasaguri Y, Hashizume T, Kanda T. When, where and how the bridge breaks: anaphase bridge breakage plays a crucial role in gene amplification and HSR generation. Exp Cell Res 2005;302:233-243.

Shiraishi K, Okita K, Kusano N, Harada T, Kondoh S, Okita S, Ryozawa S, Ohmura R, Noguchi T, Iida Y, Akiyama T, Oga A, Fukumoto Y, Furuya T, Kawauchi S, Sasaki K. A comparison of DNA copy number changes detected by comparative genomic hybridization in malignancies of the liver, biliary tract and pancreas. Oncology 2001;60:151-161.

Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. Nature 1985;315:550-554.

Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer 2003;3:807-821.

Singer MJ, Mesner LD, Friedman CL, Trask BJ, Hamlin JL. Amplification of the human dihydrofolate reductase gene via double minutes is initiated by chromosome breaks. Proc Natl Acad Sci U S A 2000;97:7921-7926.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-

2/neu oncogene. Science 1987;235:177-182.

Smith KA, Gorman PA, Stark MB, Groves RP, Stark GR. Distinctive chromosomal structures are formed very early in the amplification of CAD genes in Syrian hamster cells. Cell 1990;63:1219-1227.

Smyth GK. Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W. Bioinformatics and Computational Biology Solutions using R and Bioconductor. New York, Springer, 2005:397–420.

Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article3.

Smyth GK, Speed T. Normalization of cDNA microarray data. Methods 2003;31:265-273.

Snijders AM, Nowee ME, Fridlyand J, Piek JM, Dorsman JC, Jain AN, Pinkel D, van Diest PJ, Verheijen RH, Albertson DG. Genome-wide-array-based comparative genomic hybridization reveals genetic homogeneity and frequent copy number increases encompassing CCNE1 in fallopian tube carcinoma. Oncogene 2003;22:4281-4286.

Staal SP. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A 1987;84:5034-5037.

Stevens JL, Cantin GT, Wang G, Shevchenko A, Shevchenko A, Berk AJ. Transcription control by E1A and MAP kinase pathway via Sur2 mediator subunit. Science 2002;296:755-758.

Stratton MR, Campbell PJ, Futreal PA. The cancer genome. Nature 2009;458:719-724.

Suzuki A, Shibata T, Shimada Y, Murakami Y, Horii A, Shiratori K, Hirohashi S, Inazawa J, Imoto I. Identification of SMURF1 as a possible target for 7q21.3-22.1 amplification detected in a pancreatic cancer cell line by in-house array-based comparative genomic hybridization. Cancer Sci 2008;99:986-994.

Taatjes DJ, Marr MT, Tjian R. Regulatory diversity among metazoan co-activator complexes. Nat Rev Mol Cell Biol 2004;5:403-410.

Takagi Y, Kornberg RD. Mediator as a general transcription factor. J Biol Chem 2006;281:80-89.

Tang TC, Sham JS, Xie D, Fang Y, Huo KK, Wu QL, Guan XY. Identification of a candidate oncogene SEI-1 within a minimal amplified region at 19q13.1 in ovarian cancer cell lines. Cancer Res 2002;62:7157-7161.

Terris B, Meddeb M, Marchio A, Danglot G, Flejou JF, Belghiti J, Ruszniewski P, Bernheim A. Comparative genomic hybridization analysis of sporadic neuroendocrine tumors of the digestive system. Genes Chromosomes Cancer 1998;22:50-56.

Thompson CM, Koleske AJ, Chao DM, Young RA. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell 1993;73:1361-1375.

Thompson CM, Young RA. General requirement for RNA polymerase II holoenzymes in vivo. Proc Natl Acad Sci U S A 1995;92:4587-4590.

Thompson FH, Nelson MA, Trent JM, Guan XY, Liu Y, Yang JM, Emerson J, Adair L, Wymer J, Balfour C, Massey K, Weinstein R, Alberts DS, Taetle R. Amplification of 19q13.1-q13.2 sequences in ovarian cancer. G-band, FISH, and molecular studies. Cancer Genet Cytogenet 1996;87:55-62.

Thun MJ, DeLancey JO, Center MM, Jemal A, Ward EM. The global burden of cancer: priorities for prevention. Carcinogenesis 2010;31:100-110.

Ting AH, McGarvey KM, Baylin SB. The cancer epigenome--components and functional correlates. Genes Dev 2006;20:3215-3231.

Todd R, Wong DT. Oncogenes. Anticancer Res 1999;19:4729-4746.

Toledo F, Le Roscouet D, Buttin G, Debatisse M. Co-amplified markers alternate in megabase long chromosomal inverted repeats and cluster independently in interphase nuclei at early steps of mammalian gene amplification. EMBO J 1992;11:2665-2673.

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 2005;310:644-648.

Ueki T, Toyota M, Sohn T, Yeo CJ, Issa JP, Hruban RH, Goggins M. Hypermethylation of multiple genes in pancreatic adenocarcinoma. Cancer Res 2000;60:1835-1839.

van Dekken H, Alers JC, Riegman PH, Rosenberg C, Tilanus HW, Vissers K. Molecular cytogenetic evaluation of gastric cardia adenocarcinoma and precursor lesions. Am J Pathol 2001;158:1961-1967.

van Heek NT, Meeker AK, Kern SE, Yeo CJ, Lillemoe KD, Cameron JL, Offerhaus GJ, Hicks JL, Wilentz RE, Goggins MG, De Marzo AM, Hruban RH,Maitra A. Telomere shortening is nearly universal in pancreatic intraepithelial neoplasia. Am J Pathol 2002;161:1541-1547.

Vauhkonen H, Vauhkonen M, Sipponen P, Knuutila S. Oligonucleotide array comparative genomic hybridization refines the structure of 8p23.1, 17q12 and 20q13.2 amplifications in gastric carcinomas. Cytogenet Genome Res 2007;119:39-45.

Verjans E, Noetzel E, Bektas N, Schutz AK, Lue H, Lennartz B, Hartmann A, Dahl E, Bernhagen J. Dual role of macrophage migration inhibitory factor (MIF) in human breast cancer. BMC Cancer 2009;9:230.

Vijayvargia R, May MS, Fondell JD. A coregulatory role for the mediator complex in prostate cancer cell proliferation and gene expression. Cancer Res 2007;67:4034-4041.

Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nat Med 2004;10:789-799.

Wagner M, Luhrs H, Kloppel G, Adler G, Schmid RM. Malignant transformation of duct-like cells originating from acini in transforming growth factor transgenic mice. Gastroenterology 1998;115:1254-1262.

Wahl GM. The importance of circular DNA in mammalian gene amplification. Cancer Res 1989;49:1333-1340.

Wang P, Chen Z, Meng ZQ, Fan J, Luo JM, Liang W, Lin JH, Zhou ZH, Chen H, Wang K, Shen YH, Xu ZD, Liu LM. Dual role of Ski in pancreatic cancer cells: tumor-promoting versus metastasis-suppressive function. Carcinogenesis 2009;30:1497-1506.

Wang Y, Li Y, Zeng W, Zhu C, Xiao J, Yuan W, Wang Y, Cai Z, Zhou J, Liu M, Wu X. IXL, a new subunit of the mammalian Mediator complex, functions as a transcriptional suppressor. Biochem Biophys Res Commun 2004;325:1330-1338.

Weideman CA, Netter RC, Benjamin LR, McAllister JJ, Schmiedekamp LA, Coleman RA, Pugh BF. Dynamic interplay of TFIIA, TBP and TATA DNA. J Mol Biol 1997;271:61-75.

Weinstein IB. Cancer. Addiction to oncogenes--the Achilles heal of cancer. Science 2002;297:63-64.

Weinstein IB, Joe A. Oncogene addiction. Cancer Res 2008;68:3077-80; discussion 3080.

Windle BE, Wahl GM. Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analyses of very early events. Mutat Res 1992;276:199-224.

Yamamoto S, Tsuda H, Honda K, Onozato K, Takano M, Tamai S, Imoto I, Inazawa J, Yamada T, Matsubara O. Actinin-4 gene amplification in ovarian cancer: a candidate oncogene associated with poor patient prognosis and tumor chemoresistance. Mod Pathol 2009;22:499-507.

Yen RW, Vertino PM, Nelkin BD, Yu JJ, el-Deiry W, Cumaraswamy A, Lennon GG, Trask BJ, Celano P, Baylin SB. Isolation and characterization of the cDNA encoding human DNA methyltransferase. Nucleic Acids Res 1992;20:2287-2291.

Yin L, Velazquez OC, Liu ZJ. Notch signaling: emerging molecular targets for cancer therapy. Biochem Pharmacol 2010;80:690-701.

Yoon NK, Maresh EL, Elshimali Y, Li A, Horvath S, Seligson DB, Chia D, Goodglick L. Elevated MED28 expression predicts poor outcome in women with breast cancer. BMC Cancer 2010;10:335.

Yu W, Kanaan Y, Bae YK, Gabrielson E. Chromosomal changes in aggressive breast cancers with basal-like features. Cancer Genet Cytogenet 2009;193:29-37.

Zeng G, Germinaro M, Micsenyi A, Monga NK, Bell A, Sood A, Malhotra V, Sood N, Midda V, Monga DK, Kokkinakis DM, Monga SP. Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma. Neoplasia 2006;8:279-289.

Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A, Liang S, Naylor TL, Barchetti A, Ward MR, Yao G, Medina A, O'brien-Jenkins A, Katsaros D, Hatzigeorgiou A, Gimotty PA, Weber BL, Coukos G. microRNAs exhibit high frequency genomic alterations in human cancer. Proc Natl Acad Sci U S A 2006;103:9136-9141.

Zhang L, Maul RS, Rao J, Apple S, Seligson D, Sartippour M, Rubio R, Brooks MN. Expression pattern of the novel gene EG-1 in cancer. Clin Cancer Res 2004;10:3504-3508.

Zhu J, Woods D, McMahon M, Bishop JM. Senescence of human fibroblasts induced by oncogenic Raf. Genes Dev 1998;12:2997-3007.

Zhu Y, Qi C, Jain S, Le Beau MM, Espinosa R 3rd, Atkins GB, Lazar MA, Yeldandi AV, Rao MS, Reddy JK. Amplification and overexpression of peroxisome proliferator-activated receptor binding protein (PBP/PPARBP) gene in breast cancer. Proc Natl Acad Sci U S A. 1999;96:10848-10853

Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M. Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. Nature 2005;434:338-345.

ORIGINAL COMMUNICATIONS

Intersex-like (*IXL*) Is a Cell Survival Regulator in Pancreatic Cancer with 19q13 Amplification

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Abstract

Pancreatic cancer is a highly aggressive disease characterized by poor prognosis and vast genetic instability. Recent microarray-based, genome-wide surveys have identified multiple recurrent copy number aberrations in pancreatic cancer; however, the target genes are, for the most part, unknown. Here, we characterized the 19q13 amplicon in pancreatic cancer to identify putative new drug targets. Copy number increases at 19q13 were quantitated in 16 pancreatic cancer cell lines and 31 primary tumors by fluorescence *in situ* hybridization. Cell line copy number data delineated a 1.1 Mb amplicon, the presence of which was also validated in 10% of primary pancreatic tumors. Comprehensive expression analysis by quantitative real-time reverse transcription-PCR indicated that seven transcripts within this region had consistently elevated expression levels in the amplified versus nonamplified cell lines. High-throughput loss-of-function screen by RNA interference was applied across the amplicon to identify genes whose down-regulation affected cell viability. This screen revealed five genes whose down-regulation led to significantly decreased cell viability in the amplified PANC-1 cells but not in the nonamplified MiaPaca-2 cells, suggesting the presence of multiple biologically interesting genes in this region. Of these, the transcriptional regulator intersex-like (*IXL*) was consistently overexpressed in amplified cells and had the most dramatic effect on cell viability. *IXL* silencing also resulted in G_0-G_1 cell cycle arrest and increased apoptosis in PANC-1 cells. These findings implicate *IXL* as a novel amplification target gene in pancreatic cancer and suggest that *IXL* is required for cancer cell survival in 19q13-amplified tumors. [Cancer Res 2007;67(5):1943–9]

Introduction

Pancreatic adenocarcinoma is a highly aggressive malignancy with extremely poor prognosis. In the United States, pancreatic cancer is the fifth leading cause of cancer death, accounting for ~30,000 deaths per year (1). Pancreatic cancer is characterized by rapid progression, invasiveness, and profound resistance to treatment (2). Apart from surgery, there is practically no effective therapy; typically, the disease is diagnosed at an advanced stage when surgical resection is no longer possible. Consequently, the 5-year survival rate for pancreatic cancer is <5% and the median survival is <6 months (2, 3). Even for patients who undergo potentially curative resection, the 5-year survival rate is only ~20% (2).

Aneuploidy and increased genetic instability manifesting as complex genetic aberrations, such as losses, gains, and amplifications, are common features of pancreatic cancer (4, 5). These genetic alterations are likely to conceal genes involved in disease pathogenesis, and uncovering such genes might thus provide targets for the development of new diagnostic and therapeutic tools. In particular, gene amplification is a common mechanism for activating oncogenes, and other growth-promoting genes in cancer and amplification of target genes, such as *ERBB2* in breast cancer and *MYCN* in neuroblastomas, have been shown to have clinical significance as diagnostic and prognostic markers as well as therapeutic targets (6). We recently did a microarray-based copy number analysis in pancreatic cancer cell lines and identified an ~2.9 Mb amplicon at 19q13 (7). This result has since been confirmed in multiple subsequent studies (8–12) and the same amplicon has also been observed in other tumor types, including ovarian, breast, cervical, gastric, and small-cell lung cancer (13–20). Unfortunately, the microarray used in our previous study did not provide a complete coverage of the 19q13 region and thus did not allow direct elucidation of putative target genes. Here, we present results from the comprehensive evaluation of the 19q13 amplicon in pancreatic cancer, including detailed copy number and expression analyses as well as high-throughput loss-of-function screen using the RNA interference (RNAi) technology.

Materials and Methods

Cell lines and tissue samples. Thirteen pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAC, HPAF-II, Hs 700T, Hs 766T, MiaPaCa-2, PANC-1, SU.86.86, and SW1990, were obtained from the American Type Culture Collection (Manassas, VA). Three additional cell lines, DAN-G, HUP-T3, and HUP-T4, were acquired from the German Collection of Microorganisms and Cell Cultures (Brunswick, Germany). The cell lines were cultured under recommended conditions. Commercially available pancreatic cancer tissue microarray (AccuMaxTM Arrays) was obtained from Petagen Incorporation (Seoul, Korea). The tissue microarray contained four nonneoplastic pancreatic tissue specimens and 33 pancreatic cancer cases. Detailed clinicopathologic information on the tumor specimens is shown in Supplementary Table S1.

Genomic clones. Public genome databases (National Center for Biotechnology Information[3] and University of California Santa Cruz Genome Bioinformatics[4]) were used to select 15 bacterial artificial chromosome (BAC) clones evenly distributed over the 2.9 Mb amplicon at 19q13. These BAC clones were obtained from Invitrogen (Carlsbad, CA).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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[3] http://www.ncbi.nlm.nih.gov/

[4] http://www.genome.ucsc.edu/

In addition, a BAC clone (RP11-345J21, a kind gift from Mariano Rocchi, University of Bari, Bari, Italy) adjacent to the chromosome 19 centromere on 19q was used as a control. The list of all BAC clones is shown in Supplementary Table S2.

Fluorescence *in situ* hybridization. BAC clone DNA was labeled with SpectrumOrange-dUTP (Vysis, Downers Grove, IL) using random priming. Chromosome 19 reference probe (RP11-345J21) was labeled with fluorescein-12-dUTP (Perkin-Elmer, Boston, MA). Dual-color interphase fluorescence *in situ* hybridization (FISH) to pancreatic cancer cell lines was done as described (21). Hybridization signals were evaluated using Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan). Forty intact nuclei were scored for each probe, and relative copy numbers were calculated as ratios of mean absolute copy number of the test probe versus reference probe. FISH on tissue microarray was carried out as described (22). Three adjacent, partly overlapping BAC clones (RP11-67A5, RP11-256O9, and CTC-488F21) were combined to increase signal intensity and hybridization efficiency. Control experiments on normal lymphocytes verified that this probe combination gave a single hybridization signal. The RP11-345J21 probe was again used as a reference. Hybridization signals were scored and evaluated as described above.

Quantitative real-time reverse transcription-PCR. Gene expression analyses were done using either the Light Cycler equipment (Roche, Mannheim, Germany) or the ABI 7900HT Fast Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA was isolated from cell lines using RNeasy Mini total RNA extraction kit (Qiagen, Valencia, CA) and reverse transcribed into first-strand cDNA using Superscript III reverse transcriptase (Invitrogen) and random hexamers or iScript cDNA synthesis kit (Bio-Rad, Inc., Hercules, CA). Normal human pancreatic RNA was obtained from Ambion (Cambridgeshire, United Kingdom). For the Light Cycler analyses, primers and probe sets were obtained from TIB MolBiol (Berlin, Germany), and Light Cycler software (Roche) was used for data analysis as described (23). Expression levels of the target genes were normalized against a housekeeping gene *TBP* (TATA box binding protein; ref. 23). For the ABI system, TaqMan Gene Expression Assays were obtained from Applied Biosystems and ABI 7900HT software (Applied Biosystems) was used for data analyses. Expression levels of the target genes were normalized against an endogenous reference gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). Primer and probe sequences are shown in Supplementary Table S3.

Loss-of-function RNAi screen. Loss of function screening was done by high-throughput RNAi using a focused small interfering RNA (siRNA) library targeting all 19 known genes within the amplicon that were expressed in PANC-1 cells (Supplementary Table S4). Two genes, *SAMD4B* and *EID2B*, were hypothetical proteins at the time of the experiment and were thus not included. Four siRNAs were designed for each gene using previously described criteria (24, 25) and were obtained from Qiagen. A nonsilencing siRNA (Qiagen) was used as a negative control. High-throughput RNAi was done using siRNA reverse transfection of cells. Briefly, siRNAs were printed in quadruplicate wells of a 384-well Costar microtiter plate (Fisher Scientific, Hampton, NH). Diluted Oligofectamine (Invitrogen) was added to the wells to allow for the complexing of siRNA and transfection reagent. After a 30-min incubation period, cell suspensions of either MiaPaca-2 or PANC-1 cells were added to give a final concentration of 1,000 per well. Cells were grown at 37°C with 5% CO_2 for 96 h. Total cell number was analyzed using Cell Titer Blue (Promega, Madison WI), and the plate was read at excitation 544 nm/emission 560 nm using a EnVision plate reader (Perkin-Elmer, Wellesley, MA). Reduced cell viability to 50% or less compared with nonsilencing siRNA-treated cells was considered a significant change.

Cell cycle and apoptosis analyses. Oligofectamine reagent (Invitrogen) was used to transfect siRNAs in a final concentration of 100 nmol/L into PANC-1 cells according to the manufacturer's protocol. *IXL* 144 siRNA was used to determine the effect of inhibition of IXL expression; luciferase control siRNA, which targets the firefly *luciferase* gene (Genbank accession no. M15077), was used as a control (Supplementary Table S4). Experiments were done in triplicates using 24-well plates. After a 48-h transfection, cells were collected for cell cycle and apoptosis analyses as well as for parallel mRNA expression analyses (see above) to verify that efficient silencing was obtained. For the cell cycle analysis, trypsinized cells were centrifuged and suspended to 500 μL hypotonic staining buffer (0.1 mg/mL sodium citrate tribasic dehydrate, 0.03% Triton X-100, 50 μg/mL propidium iodide, 2 μg/mL RNase A) and the amount of propidium iodide incorporated was determined using flow cytometry (Coulter EPICS XL-MCL, Beckman Coulter, Inc., Fullerton, CA). Cell cycle distribution was analyzed using the Cylchred program.[5] Annexin V FITC Apoptosis Detection Kit (Calbiochem) was used to detect apoptotic cells by flow cytometry (Beckman Coulter, San Diego, CA).

Results

We first did a systematic characterization of the extent of the 19q13 amplicon in a panel of 16 pancreatic cancer cell lines. To this end, FISH, using 15 BAC clones evenly distributed across the 2.9 Mb region (Supplementary Table S1), was applied. Greater than 2-fold copy number increases were observed in 3 of 16 (19%) cell lines, PANC-1, Su.86.86, and HPAC (Fig. 1). The PANC-1 cell line harbored massive amplification (up to 20-fold) that on metaphase chromosomes manifested as homogeneously staining region–like structures (Fig. 1*E*), whereas lower-level copy number increases (up to 5.3- and 2.6-fold, respectively) were observed in SU.86.86 and HPAC cells (Fig. 2). The relative copy numbers of PANC-1 cells varied considerably across the 2.9 Mb region (Fig. 1*A–D*), whereas SU.86.86 and HPAC cells showed essentially uniform copy number levels across the entire amplicon. Based on the PANC-1 copy number profile, we were able to delineate a 1.1 Mb amplicon core defined by six partly overlapping BAC clones from RP11-67A5 to CTC-425O23 (Fig. 2). Furthermore, three BAC clones within this 1.1 Mb amplicon core, RP11-67A5, RP11-256O9, and CTC-488F21, displayed 14- to 20-fold copy number increase in PANC-1 cells (Fig. 2), thus defining an 660 kb subregion of extremely high level amplification.

We also examined the presence of this amplicon in primary tumors using FISH to a tissue microarray containing 33 pancreatic cancer specimens. High-level amplification (relative copy number $\geq$5) was observed in 3 of 31 tumors (9.7%) with successful hybridizations (Fig. 3). All three tumors with amplification were moderately to poorly differentiated (grades 2–3) ductal adenocarcinomas and showed lymph node metastases (Supplementary Table S2). Two of the tumor samples also had extensions to peripancreatic soft tissue with perineural invasion. The metastasis status of the third tumor was not available.

To explore the consequences of amplification on gene expression, we first used public genome databases[6] to retrieve all transcribed sequences within the core 1.1 Mb amplicon. A total of 39 transcripts, including 27 known genes and 12 hypothetical or predicted proteins, were identified (Table 1). Sixteen of these were excluded as possible candidate genes because they represented (*a*) obvious pseudogenes, (*b*) predicted transcripts with no mRNA and expressed sequence tag evidence in public databases, or (*c*) transcripts with no or very low level expression in PANC-1 (Table 1). The last criterion was based on the expectation that any putative amplification target gene should be highly expressed in PANC-1 cells that show high-level amplification. The expression levels of the remaining 23 transcripts were then assessed in all 16 pancreatic cancer cell lines using quantitative real-time RT-PCR. Interestingly, 22 of the 23 genes were highly overexpressed in

[5] http://www.cardiff.ac.uk/medicine/haematology/cytonetuk/documents/software.htm

[6] http://www.genome.ucsc.edu and http://www.ncbi.nlm.nih.gov

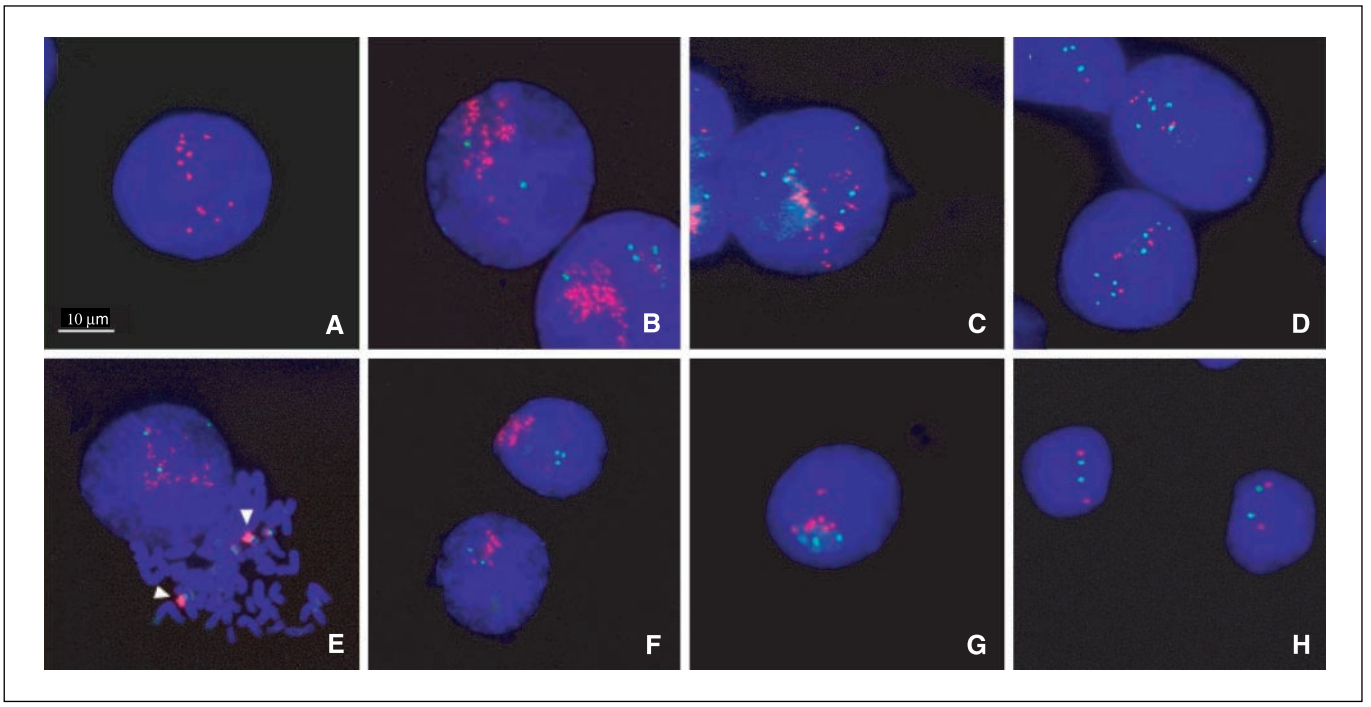

Figure 1. Copy number increases at 19q13 in pancreatic cancer cell lines. *A* to *D,* FISH signals in PANC-1 cells across the amplicon using BAC clones (*red signals*) CTC-218B8 (*A*), CTC-488F21 (*B*), RP11-246P10 (*C*), and CTC-492K19 (*D*), with a chromosome 19 pericentromeric reference probe (*green signals*). *E,* amplification manifesting as homogenously staining regions on PANC-1 metaphase chromosome (*arrows*). Two apparently normal copies of chromosome 19 are seen in the same metaphase. Low-level copy number increases in SU.86.86 (*F*) and HPAC (*G*) as well as no copy number change in Capan-1 cells (*H*). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*blue*). *Bar* (in *A*), 10 μm, for all panels.

PANC-1 cells compared with the other pancreatic cancer cell lines (Fig. 4) and normal pancreas (data not shown). Only *PLD3*, located at the distal-most end of the amplicon, was not overexpressed in PANC-1. In contrast, HPAC and Su.86.86 cells showed variable expression levels from one gene to another (Fig. 4) thus allowing us to distinguish genes differentially expressed between amplified and nonamplified cell lines. Six known genes (*GMFG, SAMD4B, IXL, SUPT5H, PSMC4*, and *MAP3K10*) and one hypothetical protein (*LOC284323*) showed consistent overexpression in all three amplified cell lines (Fig. 4). Of these, *IXL* showed the most distinct differential expression pattern between the amplified and nonamplified cell line groups, with high-level expression occurring almost exclusively in the amplified cell lines. In contrast, *GMFG* and *MAP3K10* were also highly expressed in additional cell lines with no copy number increase, indicating other activating mechanisms besides amplification. Interestingly, three of the overexpressed genes, *PSMC4, MAP3K10*, and *LOC284323*, are located outside the 660 kb amplicon maximum, thus making them less likely to be the main targets of the amplification.

To identify which of the amplified targets are functionally important, we did a targeted high-throughput RNAi screen across the defined 1.1 Mb amplicon. This loss-of-function survey was applied to study the effect of silencing of 19 known genes from the amplified region. Four different siRNAs were designed for each gene (Supplementary Table S4), and their effect on cell viability was examined in PANC-1 and MiaPaCa-2 cells 96 h after transfection. Down-regulation of *IXL* resulted in statistically highly significant reduction in cell viability ($P < 0.001$) in the amplified PANC-1 cells but not in the nonamplified MiaPaCa-2 cells (Fig. 5*A*; Supplementary Table S5). This effect was observed with two independent siRNAs in three repeated experiments. Similar reduction in cell viability was detected after *LRFN1* and *PLEKHG2* silencing as well but only with a single siRNA. Finally, down-regulation of *GMFG* and *SUPT5H* also led to reduction in cell viability but to a lesser extent (Fig. 5*A*). Other genes examined did not have a statistically significant effect on cell viability.

Further functional characterization concentrated on *IXL* because it was located within the 660 kb amplicon maximum and was implicated in both the expression survey and the RNAi viability

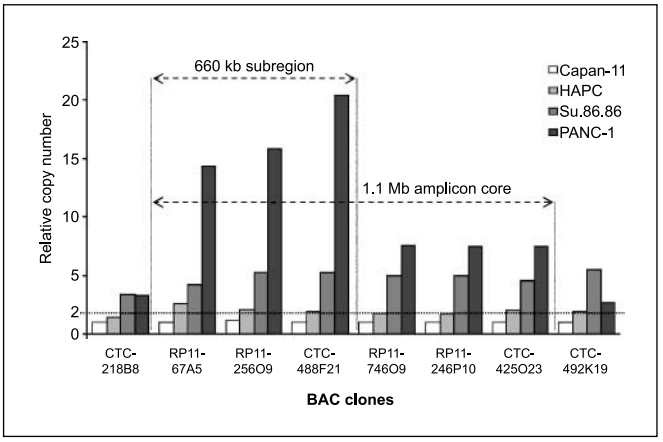

Figure 2. Amplicon mapping at 19q13 in pancreatic cancer cell lines. Relative copy number ratios for eight BAC clones around the minimal amplified region are shown for the three amplified cell lines, PANC-1, SU.86.86, and HPAC, as well as a representative nonamplified cell line, Capan-1. *Vertical dashed lines,* the 1.1 Mb core region of amplification (defined by clones RP11-67A5 and CTC-425O23) as well as the 660 kb subregion (defined by clones RP11-67A5 and CTC-488F21).

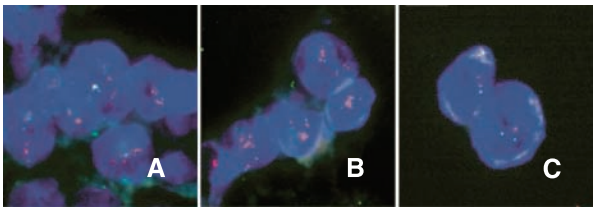

Figure 3. Copy number analysis in primary pancreatic tumors by FISH. A contig of three adjacent probes (RP11-67A5, RP11-256O9, CTC-488F21; *red signals*) was hybridized together with the chromosome 19 pericentromeric control probe (*green signals*) to a tissue microarray containing 33 pancreatic tumor samples. Examples of tumors with (*A–B*) and without (*C*) amplification.

screen. To explore the functional role of *IXL* overexpression, we transfected siRNAs targeting *IXL* into amplified PANC-1 cells and assessed the effects on cell cycle and apoptosis 48 h after transfection. A parallel mRNA expression analysis was done to verify that sufficient silencing was obtained (Fig. 5*B*). All functional experiments were done in triplicates and repeated twice. Cell cycle analysis using flow cytometry showed an increased fraction of G_0-G_1 phase cells after *IXL* siRNA (66%) transfection compared with nontransfected cells (37%) or cells transfected with a control siRNA (39%; Fig. 5*C*). Also, the percentage of apoptotic cells was increased in *IXL* siRNA–treated cells (8%) compared with control siRNA (1.6%) or untreated cells (2.5%; Fig. 5*D*). Similar phenotypic changes were not observed after *IXL* siRNA transfection to nonamplified MiaPaCa-2 cells (data not shown).

Discussion

Gross chromosomal aberrations, including losses, gains, and amplifications, are frequent in pancreatic cancer (3). Gene amplification is a common mechanism for solid tumors to upregulate the expression of genes involved in tumor progression (26, 27). Although multiple amplified regions have been documented in pancreatic carcinoma, the putative target genes activated by these aberrations are largely unknown. Identification of novel amplification target genes is extremely important because it will not only advance our knowledge on pancreatic cancer pathogenesis but it might also provide new tools for the clinical management of this highly aggressive disease.

Previously, we did a genome-wide cDNA microarray-based copy number analysis to identify localized DNA amplifications in pancreatic cancer and recognized a novel amplified region at 19q13 spanning 2.9 Mb (7). However, due to incomplete clone coverage on the microarray used, this previous study did not permit exact definition of the amplicon boundaries or direct identification of possible amplification target genes. Therefore, we now carried out a systematic evaluation of this amplified region to achieve these objectives. Three cell lines, PANC-1, SU86.86, and HPAC, harbored the 19q13 amplification, with PANC-1 cells demonstrating a massive, up to 20-fold, amplification. In addition, the copy number profile of PANC-1 allowed us to narrow down the amplicon core to 1.1 Mb, which also contained a 660 kb subregion of extremely high level amplification. Previous studies have indicated that amplification target genes are likely to be located at or near the center of the amplification maximum; that is, the region with highest copy number increase (28, 29). We thus believe this 660 kb subregion to be of particular interest in pinpointing the actual target genes of this amplicon.

Because the delineation of the amplicon core was accomplished using established pancreatic cancer cell lines, we next sought to validate the presence of the 19q13 amplicon in primary pancreatic tumors. Evaluation of a set of 31 pancreatic tumors revealed amplification in a $\sim$10% of the cases, thus confirming that this aberration is also present in actual human tumors and is not a cell culture–derived artifact. In general, oncogene amplification has been shown to be linked to advanced disease (30, 31), and, in ovarian carcinoma, 19q13 amplification has been associated with less differentiated and more aggressive tumors (14). In our series, all three tumors with amplification were moderately to poorly differentiated. All three patients had nodal metastases and two of them also had local metastases, whereas this information was missing from the third patient. Based on these tumor characteristics, the 19q13 amplification seems to be associated with advanced disease in pancreatic cancer as well. However, the number of analyzed tumors was limited; therefore, the amplification frequency as well as the possible clinicopathologic associations need to be confirmed.

A comprehensive expression analysis was subsequently done across the 1.1 Mb region to identify genes whose expression levels are elevated through 19q13 amplification. This approach was based on the well-established concept that amplification leads to increased expression of the putative target gene. Our data revealed a very distinct expression profile in PANC-1 cells demonstrating high-level expression of all but one gene throughout the entire amplicon. We hypothesize that the extremely high-level amplification in PANC-1 cells leads to complete deregulation of transcriptional control across the amplicon and thereby increased expression of all genes within this region. In contrast, SU.86.86 and HPAC cells displayed more variable expression patterns with consistent overexpression in a subset of seven genes compared with nonamplified cells, with *IXL* having the strongest association with amplification. Four of these seven genes, *GMFG, SAMD4B, IXL*, and *SUPT5H*, are located within the 660 kb amplicon maximum and were therefore considered the most likely targets.

Because the expression analysis did not explicitly pinpoint a single putative amplification target gene, we chose to perform a targeted high-throughput RNAi screen across the entire amplicon to identify functionally relevant genes. Combination of data from the expression analysis and the RNAi screen allowed us to rapidly and systematically identify genes whose expression levels were elevated through amplification, and, at the same time, whose down-regulation resulted in phenotypic changes. This strategy highlighted *IXL* as a gene that is activated by the 19q13 amplification and whose down-regulation resulted in most dramatic reduction in cell viability in amplified PANC-1 but not in nonamplified MiaPaCa-2 cells. *LRFN1* and *PLEKH2G* knockdowns also affected cell viability but not as consistently as *IXL*. However, these genes were not systematically overexpressed in all amplified cell lines. Down-regulation of *SUPT5H* and *GMFG* also depressed cell viability; however, the decrease was not as significant as for *IXL*. Based on these data, *IXL* seems to be the strongest candidate for the amplification target gene; however, we cannot exclude the involvement of some of the other genes in the region.

Further functional assays showed that *IXL* silencing resulted in increased apoptosis and G_0-G_1 arrest again in PANC-1 cells but not in MiaPaCa-2 cells, suggesting that *IXL* affects cell cycle regulatory

mechanisms that control the G_1-S transition as well as induction of apoptosis. These data implicate that *IXL* is required for cell cycle progression and cell survival in 19q13-amplified pancreatic cancer cells. *IXL* is a homologue to *Drosophila melanogaster intersex*, a transcriptional regulator involved in female somatic sex determination (32). The protein is broadly conserved during evolution (33), suggesting its importance in transcriptional regulation also in other species. Indeed, mammalian IXL has been recognized as a subunit of Mediator, a multiprotein complex that transduces regulatory signals from DNA-binding transcription factors to RNA polymerase II and thereby regulates mRNA synthesis (33, 34). The Mediator complex is required for transcriptional activation and thus controls key cellular processes. The exact function of human IXL remains elusive, although it was recently proposed to be involved in mitogen-activated protein kinase signaling pathway (33). The overexpression of IXL in pancreatic cancer may thus lead to inappropriate activation of several critical cellular processes, such as those regulating cell growth.

The *AKT2* gene has previously been suggested as the target for the 19q13 amplification (15). *AKT2* is the human homologue of the viral v-akt oncogene, which is responsible for leukemia in mice (19). Amplification of *AKT2* was originally discovered in ovarian cancer (15) but has been later observed also in other cancer types (14). Our data show that *AKT2* is indeed amplified in pancreatic cancer, although it is located at the distal-most end of the 19q13 amplicon. However, *AKT2* was not consistently overexpressed in all amplified cell lines and its down-regulation did not affect cell viability. These findings indicate that *AKT2* is clearly not the main target of the 19q13 amplification in pancreatic cancer although it cannot be ruled out that simultaneous activation of *AKT2* with other

Table 1. List of genes in the 19q13 amplicon

Gene name	Start	Stop	Description	Comment
IL29	44,478,805	44,481,152	Interleukin 29	Not expressed*
LRFN1	44,489,048	44,503,338	Leucine-rich repeat and fibronectin type III domain containing 1	
GMFG	44,510,839	44,518,460	Glia maturation factor γ	
SAMD4B	44,539,246	44,568,186	Sterile α motif domain containing 4B	
PAF1	44,568,112	44,573,519	Paf1, RNA polymerase II associated factor, homologue (*S. cerevisiae*)	
IXL	44,573,840	44,583,048	Intersex-like (*Drosophila*)	
ZFP36	44,589,327	44,591,885	Zinc finger protein 36, C3H type, homologue (mouse)	
PLEKHG2	44,595,590	44,607,994	Pleckstrin homology domain containing family G member 2	
RPS16	44,615,692	44,618,478	Ribosomal protein S16	
SUPT5H	44,628,164	44,659,150	Suppressor of Ty 5 homologue (*S. cerevisiae*)	
TIMM50	44,663,316	44,674,306	Translocase of inner mitochondrial membrane 50 homologue (yeast)	
DLL3	44,681,427	44,690,949	Delta-like 3 (*Drosophila*)	
SELV	44,539,246	44,568,186	Selenoprotein V	Not expressed*
EID-3	44,715,334	44,713,470	EID-2-like inhibitor of differentiation-3	
CRI2	44,721,289	44,722,664	CREBBP/EP300 inhibitor 2	
LOC390930	44,735,501	44,738,032	Similar to Eosinophil lysophospholipase (Charcot-Leyden crystal protein)	Pseudogene
LGALS13	44,785,004	44,789,955	Lectin, galactoside-binding, soluble, 13 (galectin 13)	Not expressed*
LOC441850	44,820,643	44,824,298	Similar to eosinophil lysophospholipase (Charcot-Leyden crystal protein)	Pseudogene
LOC148003	44,838,415	44,843,127	Similar to placental tissue protein 13 (placenta protein 13; galectin-13)	No mRNA/EST evidence
LOC400696	44,861,854	44,868,848	Eosinophil lysophospholipase-like	Not expressed*
LGALS14	44,877,533	44,891,927	Lectin, galactoside-binding, soluble, 14	Not expressed*
CLC	44,913,736	44,934,546	Charcot-Leyden crystal protein	Not expressed*
LOC342900	44,959,075	44,968,615	Hypothetical protein LOC342900	No mRNA/EST evidence
DYRK1B	45,007,831	45,016,681	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B	
FBL	45,016,938	45,028,813	Fibrillarin	
FCGBP	45,045,811	45,132,373	Fc fragment of IgG binding protein	
LOC440525	45,140,982	45,141,428	Proline-rich 13 pseudogene	Pseudogene
PSMC4	45,168,913	45,179,193	Proteasome (prosome, macropain) 26S subunit, ATPase, 4	
ZNF546	45,194,869	45,215,354	Zinc finger protein 546	
LOC390933	45,221,651	45,222,297	Similar to hypothetical protein	Pseudogene
LOC163131	45,248,079	45,232,104	Hypothetical protein LOC163131	Not evaluated
LOC284323	45,270,739	45,288,649	Hypothetical protein LOC284323	
MAP3K10	45,389,491	45,413,314	Mitogen-activated protein kinase kinase kinase 10	
TTC9B	45,413,805	45,416,138	Tetratricopeptide repeat domain 9B	Not expressed*
FLJ13265	45,419,955	45,424,404	Hypothetical protein FLJ13265	Not expressed*
LOC440526	45,429,392	45,430,177	Hypothetical protein LOC440526	
AKT2	45,431,556	45,483,036	v-akt murine thymoma viral oncogene homologue 2	
FLJ36888	45,518,813	45,546,262	Hypothetical protein FLJ36888	Not expressed*
PLD3	45,557,389	45,576,230	Phospholipase D family, member 3	

Abbreviation: EST, expressed sequence tag.
*Not expressed in PANC-1.

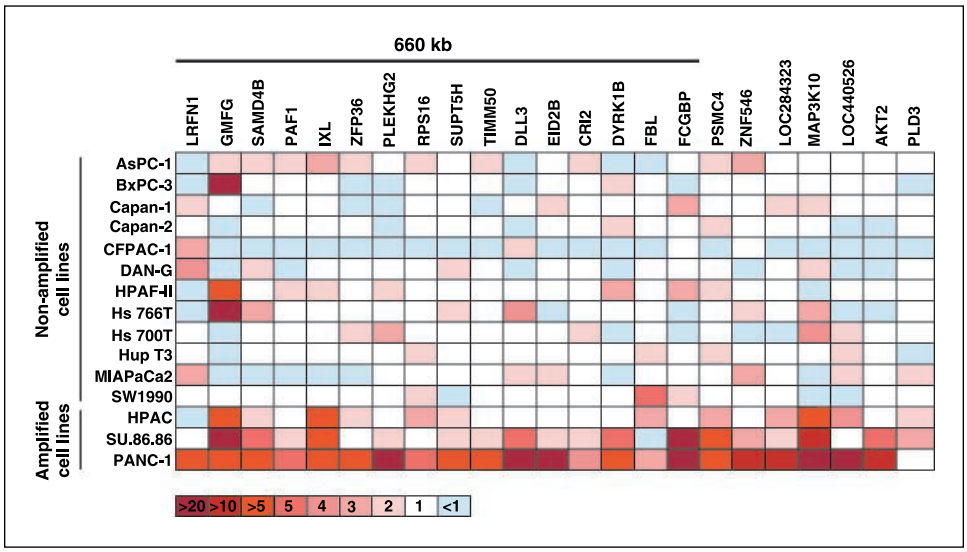

Figure 4. Schematic representation of mRNA expression levels of 23 genes within the 1.1 Mb amplicon at 19q13 in 16 pancreatic cancer cell lines. The expression levels were determined using quantitative real-time RT-PCR and were normalized against a housekeeping gene *TBP*. The relative expression levels for each gene were median centered and displayed as a pseudocolor gradient. The genes are arranged according to their chromosomal position (from centromere to telomere). *Bottom,* key to the color coding. *Horizontal line above the figure,* 660 kb amplicon core.

candidate genes from this region might provide a growth advantage for the cancer cells. Recently, two other genes from this region, *PAF1* and *DYRK1B*, were proposed to associate with pancreatic cancer development and cell survival, respectively (35, 36). Yet, again, our data do not show evidence that these genes would be the key targets of 19q13 amplicon.

In summary, our detailed characterization of the 19q13 amplicon in pancreatic cancer cell lines delineated a minimal region of amplification to a 1.1 Mb segment and further pinpointed a 660-kb amplification maximum. This amplicon was recognized in 19% of cell lines and 10% of primary pancreatic tumors. Expression profiling of genes residing in the amplicon revealed seven biologically interesting genes that were more strongly expressed in the amplified cell lines compared with the nonamplified ones. High-throughput loss-of-function screen by RNAi technology showed that down-regulation of *IXL*, and, to a lesser extent, *GMFG* and *SUPT5H*, resulted in decreased cell viability in the amplified PANC-1 but not in the nonamplified cells. Additionally, *IXL* knockdown was found to associate with G_0-G_1 arrest and increased apoptosis. Our results reveal *IXL* as a novel amplification

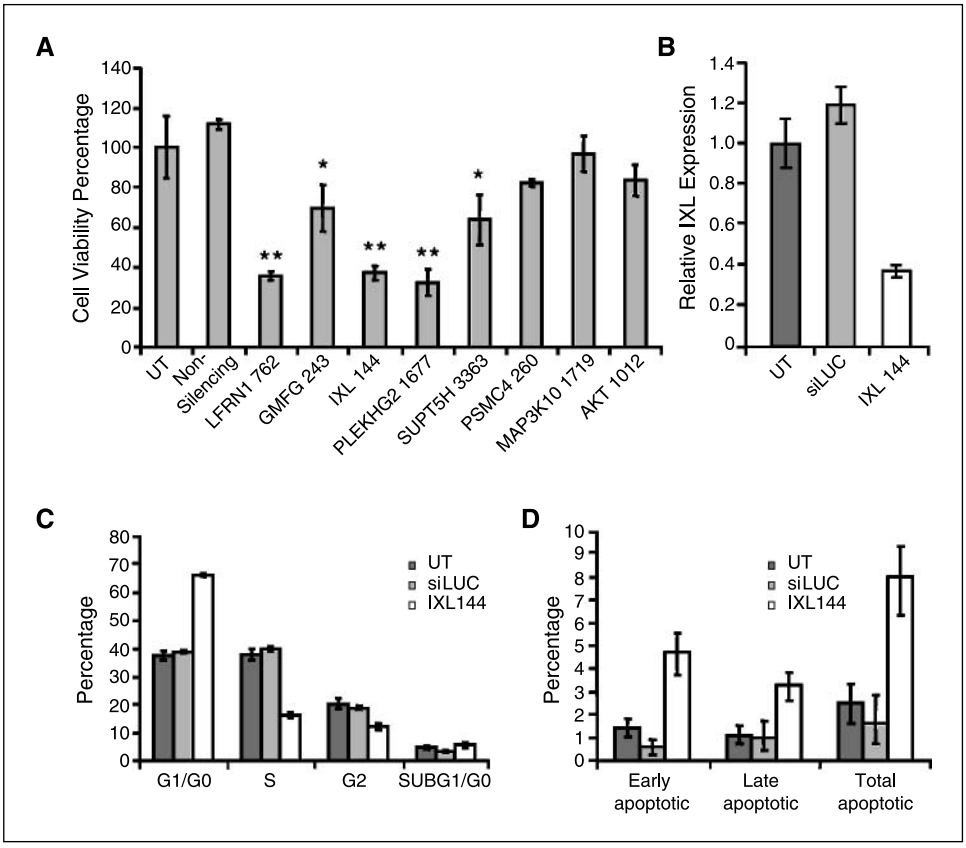

Figure 5. Functional evaluation of RNAi-based gene silencing in the PANC-1 cells. *A,* summary of data from the high-throughput RNAi viability screen. The number of cells were determined 96 h after siRNA transfection and compared with that of untreated control (*UT*). *Columns,* mean of four independent wells shown for selected genes as well as nonsilencing control siRNA; *bars,* SD. *, $P < 0.01$; **, $P < 0.001$, statistically significant reduction in cell viability. The experiment was repeated thrice with similar results. Raw data from the entire viability screen are shown in Supplementary Table S5. *B,* quantitative real-time RT-PCR analysis of *IXL* mRNA expression levels in PANC-1 cells 48 h after transfection of luciferase control siRNA (*siLUC*) or *IXL* 144 siRNA. Results were normalized against untreated cells. *Columns,* mean of triplicate experiments; *bars,* SD. *C, IXL* down-regulation results in G_0-G_1 arrest. Cell cycle analysis of untreated, luciferase siRNA–treated and *IXL* 144 siRNA–treated PANC-1 cells 48 h after transfection. *Columns,* mean of triplicate experiments; *bars,* SD. *D,* induction of apoptosis after *IXL* silencing. Percentage of early apoptotic, late apoptotic, and total apoptotic cells are shown for untreated, luciferase siRNA–treated, and *IXL* 144 siRNA–treated PANC-1 cells 48 h after transfection. *Columns,* mean of triplicate experiments; *bars,* SD.

target gene that is essential for the growth and survival of a subset of pancreatic carcinomas with 19q13 amplification. Thereby, *IXL* has a critical role in pancreatic cancer development and growth regulation and represents an ideal therapeutic target. However, it is possible that other genes in this region might also contribute to pancreatic cancer pathogenesis. Finally, this study shows that the combination of copy number and expression analysis together with targeted RNAi screen provides an efficient method for rapid identification of putative amplification target genes in cancer.

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References

1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. CA Cancer J Clin 2003; 53:5–26.

2. Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. Nat Rev Cancer 2002;2:897–909.

3. Hansel DE, Kern SE, Hruban RH. Molecular pathogenesis of pancreatic cancer. Annu Rev Genomics Hum Genet 2003;4:237–56.

4. Gorunova L, Hoglund M, Andren-Sandberg A, et al. Cytogenetic analysis of pancreatic carcinomas: intratumor heterogeneity and nonrandom pattern of chromosome aberrations. Genes Chromosomes Cancer 1998; 23:81–99.

5. Griffin CA, Hruban RH, Morsberger LA, et al. Consistent chromosome abnormalities in adenocarcinoma of the pancreas. Cancer Res 1995;55:2394–9.

6. Savelyeva L, Schwab M. Amplification of oncogenes revisited: from expression profiling to clinical application. Cancer Lett 2001;167:115–23.

7. Mahlamaki EH, Kauraniemi P, Monni O, Wolf M, Hautaniemi S, Kallioniemi A. High-resolution genomic and expression profiling reveals 105 putative amplification target genes in pancreatic cancer. Neoplasia 2004;6: 432–9.

8. Aguirre AJ, Brennan C, Bailey G, et al. High-resolution characterization of the pancreatic adenocarcinoma genome. Proc Natl Acad Sci U S A 2004;101:9067–72.

9. Bashyam MD, Bair R, Kim YH, et al. Array-based comparative genomic hybridization identifies localized DNA amplifications and homozygous deletions in pancreatic cancer. Neoplasia 2005;7:556–62.

10. Gysin S, Rickert P, Kastury K, McMahon M. Analysis of genomic DNA alterations and mRNA expression patterns in a panel of human pancreatic cancer cell lines. Genes Chromosomes Cancer 2005;44:37–51.

11. Heidenblad M, Lindgren D, Veltman JA, et al. Microarray analyses reveal strong influence of DNA copy number alterations on the transcriptional patterns in pancreatic cancer: implications for the interpretation of genomic amplifications. Oncogene 2005;24:1794–801.

12. Holzmann K, Kohlhammer H, Schwaenen C, et al. Genomic DNA-chip hybridization reveals a higher incidence of genomic amplifications in pancreatic cancer than conventional comparative genomic hybridization and leads to the identification of novel candidate genes. Cancer Res 2004;64:4428–33.

13. Arboleda MJ, Lyons JF, Kabbinavar FF, et al. Overexpression of AKT2/protein kinase bβ leads to up-regulation of β_1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. Cancer Res 2003;63:196–206.

14. Bellacosa A, de Feo D, Godwin AK, et al. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J Cancer 1995;64:280–5.

15. Cheng JQ, Godwin AK, Bellacosa A, et al. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci U S A 1992;89: 9267–71.

16. Kallioniemi A, Kallioniemi OP, Piper J, et al. Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. Proc Natl Acad Sci U S A 1994;91:2156–60.

17. Rao PH, Arias-Pulido H, Lu XY, et al. Chromosomal amplifications, 3q gain and deletions of 2q33–37 are the frequent genetic changes in cervical carcinoma. BMC Cancer 2004;4:5.

18. Ried T, Petersen I, Holtgreve-Grez H, et al. Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. Cancer Res 1994;54:1801–6.

19. Staal SP. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: Amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A 1987;84:5034–7.

20. Thompson FH, Nelson MA, Trent JM, et al. Amplification of 19q13.1-q13.2 sequences in ovarian cancer. G-band, FISH, and molecular studies. Cancer Genet Cytogenet 1996;87:55–62.

21. Barlund M, Forozan F, Kononen J, et al. Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. J Natl Cancer Inst 2000;92:1252–9.

22. Alarmo EL, Rauta J, Kauraniemi P, Karhu R, Kuukasjarvi T, Kallioniemi A. Bone morphogenetic protein 7 is widely overexpressed in primary breast cancer. Genes Chromosomes Cancer 2006;45:411–9.

23. Savinainen KJ, Linja MJ, Saramaki OR, et al. Expression and copy number analysis of TRPS1, EIF3S3 and MYC genes in breast and prostate cancer. Br J Cancer 2004;90:1041–6.

24. Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature 2004;431:343–9.

25. Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nat Biotechnol 2004;22:326–30.

26. Schwab M. Oncogene amplification in solid tumors. Semin Cancer Biol 1999;9:319–25.

27. Albertson DG, Collins C, McCormick F, Gray JW. Chromosome aberrations in solid tumors. Nat Genet 2003;34:369–76.

28. Albertson DG, Ylstra B, Segraves R, et al. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat Genet 2000;25:144–6.

29. Snijders AM, Fridlyand J, Mans DA, et al. Shaping of tumor and drug-resistant genomes by instability and selection. Oncogene 2003;22:4370–9.

30. Seeger RC, Brodeur GM, Sather H, et al. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 1985;313: 1111–6.

31. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–82.

32. Garrett-Engele CM, Siegal ML, Manoli DS, Williams BC, Li H, Baker BS. Intersex, a gene required for female sexual development in *Drosophila*, is expressed in both sexes and functions together with doublesex to regulate terminal differentiation. Development 2002; 129:4661–75.

33. Wang Y, Li Y, Zeng W, et al. IXL, a new subunit of the mammalian mediator complex, functions as a transcriptional suppressor. Biochem Biophys Res Commun 2004;325:1330–8.

34. Sato S, Tomomori-Sato C, Banks CA, et al. A mammalian homolog of *Drosophila melanogaster* transcriptional coactivator intersex is a subunit of the mammalian mediator complex. J Biol Chem 2003;278: 49671–4.

35. Moniaux N, Nemos C, Schmied BM, et al. The human homologue of the RNA polymerase II-associated factor 1 (hPaf1), localized on the 19q13 amplicon, is associated with tumorigenesis. Oncogene 2006;25:3247–57.

36. Deng X, Ewton DZ, Li S, et al. The kinase Mirk/Dyrk1B mediates cell survival in pancreatic ductal adenocarcinoma. Cancer Res 2006;66:4149–58.

19q13 Amplification Is Associated with High Grade and Stage in Pancreatic Cancer

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Pancreatic cancer is a devastating disease with an extremely poor prognosis, and thus, there is a great need for better diagnostic and therapeutic tools. The 19q13 chromosomal locus is amplified in several cancer types, including pancreatic cancer, but the possible clinical significance of this aberration remains unclear. We used fluorescence in situ hybridization on tissue microarrays containing 357 primary pancreatic tumors, 151 metastases, and 24 local recurrences as well as 120 cancer cell lines from various tissues to establish the frequency of the 19q13 amplification and to find potential correlations to clinical parameters including patient survival. Copy number increases were found in 12.2% of the primary pancreatic tumors and 9.3% of the cell lines, including those derived from bladder, colorectal, ovarian, and thyroid carcinomas. Copy number changes were linked to high grade ($P = 0.044$) and stage ($P = 0.025$) tumors, and the average survival time of patients with 19q13 amplification was shorter than that of those without this aberration. Our findings revealed recurrent 19q13 amplification in pancreatic cancer and involvement of the same locus as in bladder, colorectal, ovarian, and thyroid carcinomas. More importantly, the 19q13 amplifications were associated with poor tumor phenotype and showed a trend toward shorter survival.

INTRODUCTION

Pancreatic cancer is a lethal disease, which is typically diagnosed at an advanced stage when patients are no more eligible for curative surgery. Therefore, novel methods for early detection and better treatment of pancreatic cancer are urgently needed. Because the molecular mechanisms contributing to the development of pancreatic cancer are still not fully understood, additional information about the biology of pancreatic cancer is highly valuable and might eventually lead to the generation of novel tools for the clinical management of this disease.

Gene amplification, defined as a copy number increase of a restricted chromosomal region, is a typical mechanism for solid tumors to activate oncogenes and thereby amplified genomic regions are likely to harbor genes of importance for tumor development and progression. Excessive dosage of such genes may provide tumor cells a growth advantage or confer resistance to various therapies (Albertson, 2006). Identification of amplification target genes may offer better diagnostic and prognostic tools and even better treatment strategies. The *ERBB2* oncogene is an excellent example of an amplification target gene with well-established clinical significance as a prognostic and predictive marker and a therapeutic target in breast cancer (Carter et al., 1992). Other well-documented oncogenes that are activated via amplification and serve as prognostic markers or therapeutic targets include *MYCN* in neuroblastomas (Savelyeva and Schwab, 2001) and *EGFR* in gliomas (Etienne et al., 1998). Gene amplification has been studied widely also in pancreatic cancer, and a number of presumptive oncogenes have been pinpointed, such as *ARPC1A* (Laurila et al., 2009), *EMSY* (van Hattem et al., 2008), *GATA6* (Kwei et al., 2008), *ERBB2*, and *MYC* (Mahlamäki et al., 2002).

Genomewide comparative genomic hybridization (CGH) surveys have identified multiple regions of copy number changes in several cancers, including pancreatic cancer. They provide a powerful approach for identifying novel cancer

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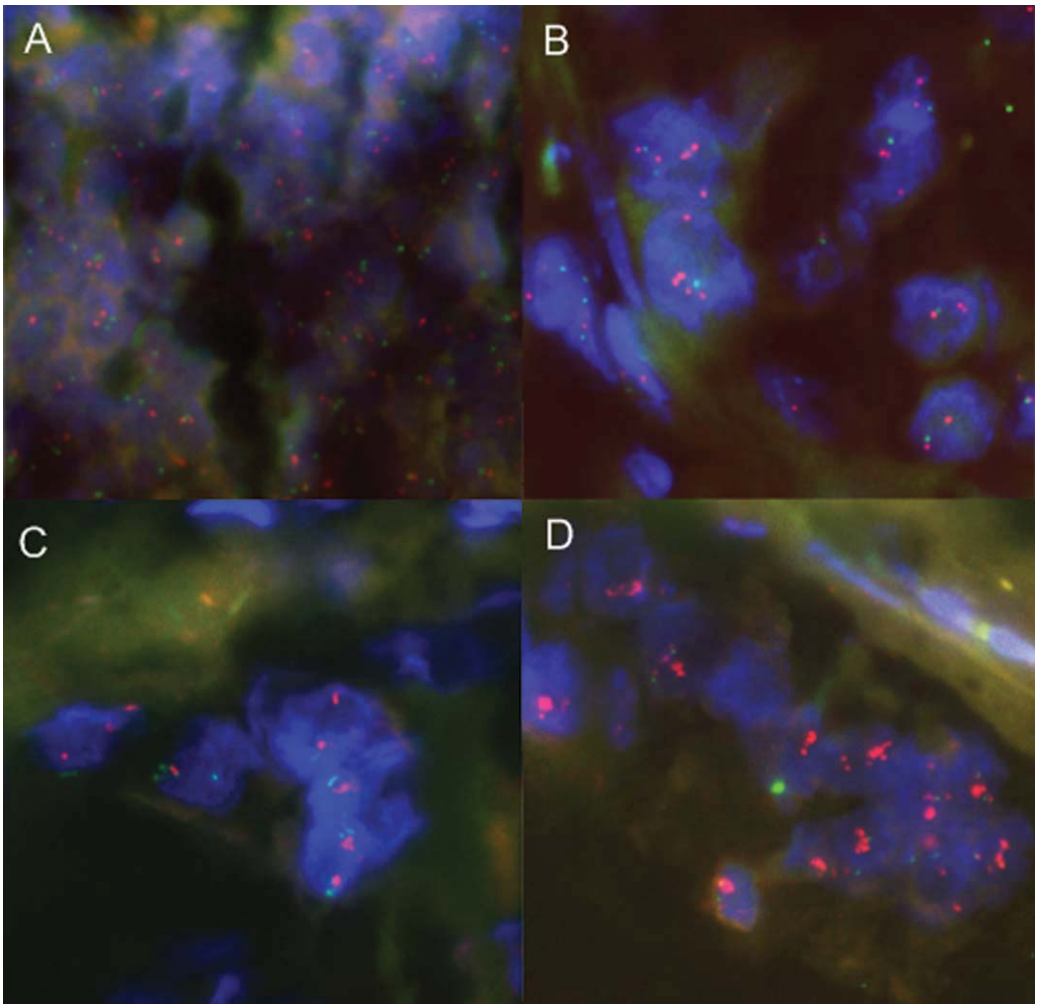

Figure 1. Analysis of 19q13 copy number levels in primary pancreatic tumors by FISH. Examples of tumors with (A) no copy number increase, (B) gain, (C) polysomy, and (D) amplification are shown. Red signals correspond to the 19q13 core region and green signals represent the Chromosome 19 centromere probe. Nuclei were counterstained with DAPI (blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

genes when combined with further genetic and functional studies. Our previous array CGH screen (Mahlamäki et al., 2004) revealed a 2.9-Mb region at 19q13 that is recurrently amplified in pancreatic cancer. This finding has been subsequently confirmed by several other microarray-based copy number surveys (Aguirre et al., 2004; Holzmann et al., 2004; Bashyam et al., 2005; Gysin et al., 2005; Heidenblad et al., 2005). Recently, we characterized the structure and boundaries of the 19q13 amplicon in detail in pancreatic cancer cell lines and defined a 660-kb amplicon core region with exceptionally high level copy number increase (Kuuselo et al., 2007). This chromosomal region is extremely gene-rich (Supporting Information Fig. 1). Only a subset of the genes within this region, such as *MED29* (previously known as *IXL*), *PAF1*, *DYRK1B*, and *PAK4*, have been functionally validated and suggested to represent putative amplicon targets (Kuuselo et al., 2007; Moniaux et al., 2006; Deng et al., 2006; Chen et al., 2008).

In addition to pancreatic cancer, amplification of the 19q13 chromosomal region has also been reported in other tumor types, such as ovarian (Cheng et al., 1992; Bellacosa et al., 1995; Thompson et al., 1996; Tang et al., 2002), breast (Kallioniemi et al., 1994; Bellacosa et al., 1995), cervical (Rao et al., 2004), gastric (Staal, 1987), and lung cancer (Ried et al., 1994; Kim et al., 2005). However, these studies have been performed using a variety of technologies, ranging from chromosomal CGH to different array CGH platforms, and thus, it is difficult to know

whether they pinpoint a single common amplicon or correspond to multiple separate regions of copy number increase at 19q13. Here, we examined for the first time the presence of copy number aberrations at the 660-kb amplicon core (Kuuselo et al., 2007) in 120 cancer cell lines originating from various tissues.

In a previous study, we demonstrated using a small set of 31 tumors that the 19q13 amplification is present in about 10% of the primary pancreatic cancer cases (Kuuselo et al., 2007). Now, we evaluated the clinical significance of the 19q13 amplicon core in an extensive sample set containing more than 500 pancreatic tumors. We applied fluorescence in situ hybridization (FISH) to a tissue microarray (TMA) containing 357 primary tumors of the pancreas, 151 metastases, and 24 local recurrences to determine the 19q13 copy number levels and to reveal their possible association with clinicopathological parameters and patient survival.

MATERIALS AND METHODS

Patients with Pancreatic Cancer

Primary pancreatic tumor samples and corresponding metastases were obtained from 356 patients who underwent pancreatic surgery at the Department of General, Visceral and Thoracic Surgery, University Medical Center Hamburg-Eppendorf, during the years 1993–2005. Formalin-fixed (buffered neutral aqueous 4% solution) paraffin-embedded material was used. All slides from all tumors were reviewed by two pathologists determining the histological type and grade of the samples (G1 = highly differentiated, G2 = moderately differentiated, and G3 = poorly differentiated). The pathologic stage, nodal status, and metastasis information were obtained from the primary reports of the Department of Pathology, University Medical Center Hamburg-Eppendorf. Follow-up and survival data were collected by the Department of General, Visceral and Thoracic Surgery. The following TNM classification was used: pT stage, T1 = tumor size $\leq$ 2 cm, T2 = tumor size $>$ 2 cm, T3 = tumor growth into surrounding tissues, T4 = tumor growth into the stomach, spleen, large bowel, or nearby large blood vessels; pN stage, N0 = no lymph node metastases, N1 = metastases in local lymph nodes; and pM stage, M0 = no distant metastases, M1 = distant metastases. Median age of the patients was 62.8 years (range 21–88 years). The mean follow-up time for ductal adenocarcinomas was 18.52 months (range 1–74 month). Informed consent had been obtained from all patients upon admission to hospital.

Tissue Microarrays

The pancreatic cancer tissue microarray (TMA) contained a total of 600 samples. These included 357 primary tumors of the pancreas [213 ductal adenocarcinomas, 54 adenocarcinomas of the ampulla of Vater, 40 pancreatic endocrine tumors, 33 intraductal papillary mucinous neoplasms (IPMNs), 15 benign cystic tumors, 1 malignant cystic tumor, and 1 acinar cell carcinoma], 129 corresponding lymph node metastases, 22 distant metastases, 24 local recurrences, and a standard control area containing 40 tumors from other organs, 10 healthy pancreatic tissues, and 18 healthy tissues from other sites. The cell line TMA had 120 cancer cell lines representing various tissue types and nine cell lines of non-neoplastic origin (Supporting Information Table 1).

Fluorescence In Situ Hybridization

To determine the 19q13 copy number levels, we used a contig of three partly overlapping locus-specific BAC probes (RP11-67A5, RP11-256O9, and CTC-488F21) that were previously shown to correspond to the 660-kb core region of the amplicon (Supporting Information Fig. 1) and verified to give a single signal on normal lymphocytes (Kuuselo et al., 2007). The BAC clone DNA was isolated using standard alkaline lysis method and labeled with Spectrum Orange dUTP (Vysis, Downers Grove, IL) using random priming. A chromosome 19 pericentromere-specific reference probe (RP11-345J21) was labeled with fluorescein-12-dUTP (PerkinElmer Life Sciences, Boston, MA) and used as a control. FISH on TMA was carried out as described (Andersen et al., 2001) with some modifications. Briefly, the slides were deparaffinized in three changes of hexane for 10 min each, dipped twice in 100% ethanol, treated for 30 min with 0.3% $NaBH_4$, and rinsed with PBS. Then the slides were treated for 40 min with Vysis Pretreatment Solution (Vysis, Downers Grove, IL) at 80°C, rinsed with H_2O, and treated for 20 min with Vysis Protease at 37°C, followed by Proteinase K treatment for 10 min at 37°C. Finally, the slides were washed in increasing series of ethanol (70, 85, and 100%), dehydrated, denatured for 3 min

TABLE 1. Primary Pancreatic Cancer Subtypes and Their Copy Number Status

		19q13 FISH result				
	n	Analyzable (*n*)	Normal (%)	Gain (%)	Amplification (%)	Polysomy (%)
All samples	357	303	87.8	7.3	3.3	1.7
Ductal adenocarcinomas	213	197	86.3	8.1	3.6	2.0
Intraductal papillary mucinous neoplasms	33	15	93.3	0.0	6.7	0.0
Endocrine tumors	40	35	88.6	8.6	0.0	2.9
Cystic/benign tumors	15	6	100	0.0	0.0	0.0
Cystic/malignant tumors	1	1	100	0.0	0.0	0.0
Acinar cell cancers	1	0	–	–	–	–
Adenocarcinomas of the ampulla of Vater	54	49	89.8	6.1	4.1	0.0
Metastases	151	121	94.2	3.3	2.5	0.0
Lymph node metastases	129	100	95.0	3.0	2.0	0.0
Distant metastases	22	21	90.4	4.8	4.8	0.0
Local recurrences	24	18	100	0.0	0.0	0.0

at 70°C in 70% formamide/2× SSC, washed again in ethanol series, dehydrated, and hybridized with denatured probes in a humidified chamber at 37°C for 24 hr. The nuclei were counterstained with DAPI in Vectashield antifade solution.

Hybridization signals were evaluated using Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan), and relative copy numbers were calculated for each sample as ratios of mean absolute copy number of the locus-specific probe versus the reference probe. Forty intact nuclei were scored per sample. Relative copy numbers over 1.5 but less than 2 were considered as gains, whereas relative copy numbers greater than or equal to 2 were considered as amplifications. Polysomy was defined as samples with absolute copy number of the 19q13 locus over five but relative copy number less than two.

Statistical Analyses

Statistical analyses were done using JMP™ software (SAS Institute, Cary, NC). All P values tested were two-sided and $P < 0.05$ was considered significant. The Pearson χ^2 test was used to assess the relationship between 19q13 copy number changes (categorized as normal, gain, amplification, and polysomy) and the clinicopathologic parameters T stage, *n* stage, and tumor grade. The Kaplan–Meier method was used to visualize association of 19q13 copy number changes with cancer-specific survival, and the log rank test was applied to test the significance between stratified groups.

RESULTS

The presence of copy number changes at the 19q13 amplicon core was first screened in a set of 120 cancer cell lines representing various tumor types (Supporting Information Table 1). Ten (9.3%) of the 107 cell lines with successful hybridizations displayed increased copy number. Six of these were amplifications (relative copy number ≥ 2) and four were gains (relative copy number >1.5 but <2). Amplifications were detected in one (OVCAR-3) of four ovarian cancer cell lines, three (RT-112, KU-19-19, CRL-7930) of six bladder, one (SW-48) of twelve colorectal cell lines, and one (ONCO-DG-1) of four thyroid carcinoma cell lines.

We then determined the frequency of the 19q13 copy number changes in 357 primary pancreatic tumors, 151 metastatic lesions (including both lymph node and distant metastases), and 24 local recurrences. Among the primary tumors, copy number data were obtained in 303 cases and 12.2% of them had copy number increases (Fig. 1). Gains were detected in 7.3%, amplifications in 3.3%, and polysomy in 1.7% of the cases. Copy number changes were most frequently observed in ductal adenocarcinomas and pancreatic endocrine tumors (Table 1). In addition, copy number increase was also detected in one of 15 IPMNs. Tumor samples with amplification typically showed a tight cluster of signals with an average of 3.4-fold copy number increase, but a few cases with up to 10-fold amplification levels were observed. The polysomic samples had an average of five to ten copies of the 19q13 locus per cell, ranging up to twenty copies.

A total of seven (5.8%) of the 121 metastases with successful hybridizations showed copy number increase (Table 1). Of these, three were amplifications and four cases had gains. Copy number data were available on a subset of 91 cases where both the primary tumor and a

TABLE 2. Relationship Between the 19q13 Copy Number Changes and the Cancer Phenotype in Pancreatic Ductal Adenocarcinomas

		n	Normal (%)	Gain (%)	Amplification (%)	Polysomy (%)	*P*
pT stage	pT1	6	83.3	0.0	0.0	16.7	0.025[a]
	pT2	53	92.5	1.9	1.9	3.8	
	pT3	125	84.8	10.4	4.0	0.8	
	pT4	9	66.7	22.2	11.1	0.0	
pN stage	pN0	68	83.8	10.3	4.4	1.5	0.826
	pN1	124	87.1	7.3	3.2	2.4	
Grade	G1	8	100	0.0	0.0	0.0	0.044[b]
	G2	91	89.0	9.9	0.0	1.1	
	G3	95	83.2	7.4	7.4	2.1	

[a](T1 + T2 vs. T3 + T4).
[b](G1 + G2 vs. G3).

matching lymph node metastasis were studied. Among this subset of cases, increased copy number was detected in four primary tumors and three corresponding metastases thus showing an overall concordance of 99% (90/91). Finally, none of the local recurrences (of a total of 18 analyzable cases) showed copy number increases (Table 1). However, 17 of the 18 corresponding primary tumors also had no copy number change, while the one remaining case did show copy number gain.

To evaluate the possible clinical significance of the 19q13 amplification, the relationships between copy number data and clinicopathological characteristics were examined among the ductal adenocarcinomas, the most common histological subtype of pancreatic cancer (Table 2). Because of the small number of samples, tumors confined to pancreas (pT1 and pT2) were compared to those that had spread beyond the pancreas (pT3 and pT4). Similarly, moderately and well-differentiated tumors (G1 and G2) were combined and compared to poorly differentiated tumors (G3). Copy number increases (including gains, amplifications, and polysomy) were linked to both tumor grade and stage ($P = 0.044$ and $P = 0.025$, respectively, Table 2). The frequency of gains and amplifications increased from low/moderate (G1–G2) to high grade (G3) tumors and from early (pT1–pT2) to late (pT3–pT4) stage tumors. Actually none of the low-grade (G1) tumors harbored copy number changes, whereas 11% of Grade 2 tumors and 16.8% of Grade 3 tumors had increased copy number. All of the tumors with amplification were of Grade 3.

Finally, the Kaplan–Meier analysis among the ductal adenocarcinomas showed that the survival of patients having high level copy number changes (amplifications and polysomy) was somewhat worse than those without 19q13 copy number changes, but this difference was not statistically significant (data not shown). Nonetheless, the average survival time for the normal copy number patient group was 26 months, whereas it was only 16 and 17 months for the patients with amplification and polysomy, respectively.

DISCUSSION

Pancreatic cancer is a highly devastating disease with exceptionally poor prognosis. Consequently, there is a huge need for better understanding of the biology of this disease and for identification of novel diagnostic and prognostic markers as well as molecular targets for therapy. Every piece of knowledge about the mechanisms behind pancreatic cancer development may shift us toward novel strategies for improved clinical applications. To this end, we evaluated the clinical significance of the 19q13 amplification that was initially discovered by us and others using genomewide copy number screens of pancreatic cancer (Mahlamäki et al., 2004; Aguirre et al., 2004; Holzmann et al., 2004; Bashyam et al., 2005; Gysin et al., 2005; Heidenblad et al., 2005). Recently, we characterized this amplicon in detail in pancreatic cancer cell lines and in a small set of primary pancreatic tumors and defined a 660-kb amplicon core region (Kuuselo et al., 2007). Here, we studied the clinical significance of the 19q13 amplification in a large collection of over 500 clinical pancreatic tumor samples using FISH to TMAs. We also examined whether this specific amplicon is present in other tumor types using more than 100 different cancer cell lines. The FISH technique is perhaps the most reliable and accurate method to detect

different types of copy number changes from chromosomal rearrangements to amplifications and deletions (Fletcher, 1999). It now represents a standard diagnostic tool in the classification of hematological malignancies and in detecting clinically relevant gene amplification events, such as those involving the *ERBB2* oncogene in breast cancer (Spiridon et al., 2002).

Copy number analysis in an extensive collection of over 500 pancreatic tumors revealed that 12.2% of the primary cancers had 19q13 copy number increases. This frequency is in good concordance with our preliminary data from a small set of 31 tumors (Kuuselo et al., 2007). The copy number aberrations were divided into three categories: gains were detectable in 7.3% of the cases, amplification in 3.3%, and 1.7% of the tumors were polysomic. Moreover, a concordant copy number result between a primary tumor and a corresponding lymph node metastasis was obtained in 99% of the samples. Surprisingly, none of the local recurrences showed copy number changes. However, the number of samples analyzed was small and more importantly the corresponding primaries did not show copy number aberrations either. Thus, these data do not allow us to draw conclusions on the possible genetic differences between the primaries and local recurrences.

Although amplification of the 19q13 chromosomal locus has been previously reported in a subset of other cancer types, here we characterized for the first time the presence of this specific amplicon core in a large number of cell lines representing various tumor types. In addition to pancreatic tumors, we found amplification of the 19q13 core region in ovarian, colorectal, urinary bladder, and thyroid cancer cell lines. These data now confirm previous studies in ovarian (Bellacosa et al., 1995; Cheng et al., 1992; Thompson et al., 1996; Tang et al., 2002), colorectal (Bardi et al., 1993), and urinary bladder (Richter et al., 2000) cancer. However, 19q13 amplification has not been previously reported in thyroid cancer. The cell line TMA data now allow rapid identification of numerous cell lines that are suitable for further functional analyses of putative target genes within the 19q13 amplicon.

Gene amplification has been shown to frequently associate with tumor progression, drug resistance, and poor clinical outcome in a variety of tumor types (Savelyeva and Schwab, 2001). For example, amplification of oncogenes, such as *CCND1*, *EGFR*, *ERBB2*, *MDM2*, and *MYC*, has been associated with high grade breast tumors (Al-Kuraya et al., 2004), *MYCN* amplification is a prognostic factor for patients with neuroblastoma (Savelyeva and Schwab, 2001), and amplification of genes such as *DHFR* and *BCR-ABL1* has been reported to be associated with resistance to anticancer drugs (Albertson, 2006). Now we found that the 19q13 copy number increases were linked to both advanced tumor stage and grade in pancreatic cancer. Similarly, 19q13 amplification has been previously shown to associate with less differentiated and more aggressive tumors in ovarian carcinoma (Bellacosa et al., 1995).

Our results suggest a trend toward shorter survival time in patients with 19q13 amplification. The average survival time of patients with high level copy number increases (amplification or polysomy) was shorter than the survival time of patients with normal copy number status, although this difference was not statistically significant. The lack of statistical significance may be due to the rather small number of tumors with copy number changes but also on the overall poor prognosis of pancreatic cancer. In any case, our data are in concordance with the finding that high level copy number increases have a greater impact on tumor development than low level aberrations (Hodgson et al., 2003). Previously, the 19q13 amplification has been associated with poor survival of patients with non-small cell lung cancers (Kim et al., 2005), but there are no other studies reporting associations of this specific amplification locus with patient survival. However, the connection of the 19q13 amplification to more aggressive and less differentiated tumors (Bellacosa et al., 1995; Tang et al., 2002) could support the hypothesis that this amplicon is indeed associated with poor prognosis.

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REFERENCES

Aguirre AJ, Brennan C, Bailey G, Sinha R, Feng B, Leo C, Zhang Y, Zhang J, Gans JD, Bardeesy N, Cauwels C, Cordon-Cardo C, Redston MS, DePinho RA, Chin L. 2004. High-resolution characterization of the pancreatic adenocarcinoma genome. Proc Natl Acad Sci USA 101:9067–9072.

Albertson DG. 2006. Gene amplification in cancer. Trends Genet 22:447–455.

Al-Kuraya K, Schraml P, Torhorst J, Tapia C, Zaharieva B, Novotny H, Spichtin H, Maurer R, Mirlacher M, Kochli O, Zuber M, Dieterich H, Mross F, Wilber K, Simon R, Sauter G. 2004. Prognostic relevance of gene amplifications and coamplifications in breast cancer. Cancer Res 64:8534–8540.

Andersen CL, Hostetter G, Grigoryan A, Sauter G, Kallioniemi A. 2001. Improved procedure for fluorescence in situ hybridization on tissue microarrays. Cytometry 45:83–86.

Bardi G, Johansson B, Pandis N, Mandahl N, Bak-Jensen E, Lindstrom C, Tornqvist A, Frederiksen H, Andren-Sandberg A, Mitelman F. 1993. Cytogenetic analysis of 52 colorectal carcinomas—Non-random aberration pattern and correlation with pathologic parameters. Int J Cancer 55:422–428.

Bashyam MD, Bair R, Kim YH, Wang P, Hernandez-Boussard T, Karikari CA, Tibshirani R, Maitra A, Pollack JR. 2005. Array-based comparative genomic hybridization identifies localized DNA amplifications and homozygous deletions in pancreatic cancer. Neoplasia 7:556–562.

Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V. 1995. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J Cancer 64:280–285.

Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM. 1992. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci USA 89:4285–4289.

Chen S, Auletta T, Dovirak O, Hutter C, Kuntz K, El-Ftesi S, Kendall J, Han H, Von Hoff DD, Ashfaq R, Maitra A, Iacobuzio-Donahue CA, Hruban RH, Lucito R. 2008. Copy number alterations in pancreatic cancer identify recurrent PAK4 amplification. Cancer Biol Ther 7.

Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, Testa JR. 1992. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci USA 89:9267–9271.

Deng X, Ewton DZ, Li S, Naqvi A, Mercer SE, Landas S, Friedman E. 2006. The kinase Mirk/Dyrk1B mediates cell survival in pancreatic ductal adenocarcinoma. Cancer Res 66:4149–4158.

Etienne MC, Formento JL, Lebrun-Frenay C, Gioanni J, Chatel M, Paquis P, Bernard C, Courdi A, Bensadoun RJ, Pignol JP, Francoual M, Grellier P, Frenay M, Milano G. 1998. Epidermal growth factor receptor and labeling index are independent prognostic factors in glial tumor outcome. Clin Cancer Res 4:2383–2390.

Fletcher JA. 1999. DNA in situ hybridization as an adjunct in tumor diagnosis. Am J Clin Pathol 112:S11–S18.

Gysin S, Rickert P, Kastury K, McMahon M. 2005. Analysis of genomic DNA alterations and mRNA expression patterns in a panel of human pancreatic cancer cell lines. Genes Chromosomes Cancer 44:37–51.

Heidenblad M, Lindgren D, Veltman JA, Jonson T, Mahlamaki EH, Gorunova L, van Kessel AG, Schoenmakers EF, Hoglund M. 2005. Microarray analyses reveal strong influence of DNA copy number alterations on the transcriptional patterns in pancreatic cancer: implications for the interpretation of genomic amplifications. Oncogene 24:1794–1801.

Hodgson JG, Chin K, Collins C, Gray JW. 2003. Genome amplification of chromosome 20 in breast cancer. Breast Cancer Res Treat 78:337–345.

Holzmann K, Kohlhammer H, Schwaenen C, Wessendorf S, Kestler HA, Schwoerer A, Rau B, Radlwimmer B, Dohner H, Lichter P, Gress T, Bentz M. 2004. Genomic DNA-chip hybridization reveals a higher incidence of genomic amplifications in pancreatic cancer than conventional comparative genomic hybridization and leads to the identification of novel candidate genes. Cancer Res 64:4428–4433.

Kallioniemi A, Kallioniemi OP, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW, Waldman FM. 1994. Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. Proc Natl Acad Sci USA 91:2156–2160.

Kim TM, Yim SH, Lee JS, Kwon MS, Ryu JW, Kang HM, Fiegler H, Carter NP, Chung YJ. 2005. Genome-wide screening of genomic alterations and their clinicopathologic implications in non-small cell lung cancers. Clin Cancer Res 11:8235–8242.

Kuuselo R, Savinainen K, Azorsa DO, Basu GD, Karhu R, Tuzmen S, Mousses S, Kallioniemi A. 2007. Intersex-like (IXL) is a cell survival regulator in pancreatic cancer with 19q13 amplification. Cancer Res 67:1943–1949.

Kwei KA, Bashyam MD, Kao J, Ratheesh R, Reddy EC, Kim YH, Montgomery K, Giacomini CP, Choi YL, Chatterjee S, Karikari CA, Salari K, Wang P, Hernandez-Boussard T, Swarnalata G, van de Rijn M, Maitra A, Pollack JR. Genomic profiling identifies GATA6 as a candidate oncogene amplified in pancreatobiliary cancer. 2008. PLoS Genet 4:e1000081.

Laurila E, Savinainen K, Kuuselo R, Karhu R, Kallioniemi A. 2009. Characterization of the 7q21–q22 amplicon identifies ARPC1A, a subunit of the Arp2/3 complex, as a regulator of cell migration and invasion in pancreatic cancer. Genes Chromosomes Cancer 48:330–339.

Mahlamäki EH, Bärlund M, Tanner M, Gorunova L, Höglund M, Karhu R, Kallioniemi A. 2002. Frequent amplification of 8q24, 11q, 17q, and 20q-specific genes in pancreatic cancer. Genes Chromosomes Cancer 35:353–358.

Mahlamäki EH, Kauraniemi P, Monni O, Wolf M, Hautaniemi S, Kallioniemi A. 2004. High-resolution genomic and expression profiling reveals 105 putative amplification target genes in pancreatic cancer. Neoplasia 6:432–439.

Moniaux N, Nemos C, Schmied BM, Chauhan SC, Deb S, Morikane K, Choudhury A, Vanlith M, Sutherlin M, Sikela JM, Hollingsworth MA, Batra SK. 2006. The human homologue of the RNA polymerase II-associated factor 1 (hPaf1), localized on the 19q13 amplicon, is associated with tumorigenesis. Oncogene 25:3247–3257.

Rao PH, Arias-Pulido H, Lu XY, Harris CP, Vargas H, Zhang FF, Narayan G, Schneider A, Terry MB, Murty VV. 2004. Chromosomal amplifications, 3q gain and deletions of 2q33–q37 are the frequent genetic changes in cervical carcinoma. BMC Cancer 4:5.

Richter J, Wagner U, Kononen J, Fijan A, Bruderer J, Schmid U, Ackermann D, Maurer R, Alund G, Knonagel H, Rist M, Wilber K, Anabitarte M, Hering F, Hardmeier T, Schonenberger A, Flury R, Jager P, Fehr JL, Schraml P, Moch H, Mihatsch MJ, Gasser T, Kallioniemi OP, Sauter G. 2000. High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. Am J Pathol 157:787–794.

Ried T, Petersen I, Holtgreve-Grez H, Speicher MR, Schrock E, du Manoir S, Cremer T. 1994. Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. Cancer Res 54:1801–1806.

Savelyeva L, Schwab M. 2001. Amplification of oncogenes revisited: From expression profiling to clinical application. Cancer Lett 167:115–123.

Spiridon CI, Ghetie MA, Uhr J, Marches R, Li JL, Shen GL, Vitetta ES. 2002. Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antigrowth activity of a human breast cancer cell line in vitro and in vivo. Clin Cancer Res 8:1720–1730.

Staal SP. 1987. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: Amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci USA 84:5034–5037.

Tang TC, Sham JS, Xie D, Fang Y, Huo KK, Wu QL, Guan XY. 2002. Identification of a candidate oncogene SEI-1 within a minimal amplified region at 19q13.1 in ovarian cancer cell lines. Cancer Res 62:7157–7161.

Thompson FH, Nelson MA, Trent JM, Guan XY, Liu Y, Yang JM, Emerson J, Adair L, Wymer J, Balfour C, Massey K, Weinstein R, Alberts DS, Taetle R. 1996. Amplification of 19q13.1–q13.2 sequences in ovarian cancer. G-band, FISH, and molecular studies. Cancer Genet Cytogenet 87:55–62.

vanHattem WA, Carvalho R, Li A, Offerhaus GJ, Goggins M. 2008. Amplification of EMSY gene in a subset of sporadic pancreatic adenocarcinomas. Int J Exp Pathol 1:343–351.